Preparation and Characterization of Ergocalciferol-Loaded Nanodispersions Stabilized by Different Emulsifiers

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Gaofeng SHU

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A Dissertation Submitted to

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Gaofeng SHU

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Abbreviations

EE	Encapsulation efficiency
EPI	Emulsion phase inversion
FFA	Free fatty acids
HPLC	High-performance liquid chromatography
HLB	Hydrophilic-lypophilic balance
MCT	Medium chain triglycerides
ML	Modified lecithin
MO7S	Decaglycerol monooleate
O/W	Oil-in-water emulsions
PGEs	Polyglycerol esters of fatty acids
PDI	Polydispersity index
PI	Isoelectric point
PIT	Phase inversion temperature
SC	Sodium caseinate
SGF	Simulated gastric fluid
SSF	Simulated saliva fluid
W/O	Water-in-oil

Chapter 1

Introduction

1.1 Background

In recent years, the increase in age and diet related diseases, such as obesity, diabetes, hypertension, hyperglycemia, cardiovascular diseases and cancers, has become a prominent problem in many countries. It is widely accepted that the dairy intake of bioactive components are supposed to decrease risks of aforementioned illness and improve life conditions. In this regard, people pay more attention on food products containing functional components, which satisfy hunger but also offer additional health benefits, such as anti-cancer, and anti-oxidant abilities. This trend encourage food scientists and manufacturers to focus on the production of foods that meet consumers' criteria for healthier lives (Bigliardi and Galati 2013).

However, many bioactive components, (e.g., curcumin, carotenoids, vitamins, quercetin, and polyunsaturated oils *etc.*) are hydrophobic and prone to degradation against harsh conditions (e.g., high temperature, light, and oxygen), which strongly limit their potential application in fortifying aqueous-based beverages and foods. Furthermore, low water-solubility also means low absorption by the human body and limited bioavailability. To overcome these challenges, an edible delivery system is urgently needed to effectively encapsulate, protect and release the bioactive components when developing functional foods. Currently, nanotechnology plays an important role in food manufacturing, including nano-delivery systems for bioactives, health-promoting products and so on. The value of food products produced by nanotechnology is expected to be more than US$ 20.4 billion in a few years (Chau *et al.*, 2007). A number of evidences have shown that nanoemulsions are excellent candidates for effectively delivering those functional lipophilic components due to their important role in increasing water-solubility and bioaccessibility (Salvia-Trujillo *et al.*, 2015, Zhang *et al.*, 2016, Ahmed *et al.*, 2012, Yang

et al., 2017). The bioaccessibility of lipophilic bioactives encapsulated in nanoemulsions is commonly greater than conventional emulsions, which may be attributed to a number of reasons (McClements 2011, McClements 2013, Acosta 2009), such as: (1) Smaller droplets size means larger surface area, which leads to a faster lipid digestion to form mixed micelles for solubilizing bioactives; (2) Smaller particles can also penetrate into the mucus layer in small intestine, which increase the time for lipid digestion and absorption; (3) Some of small droplets can be directly absorbed by epithelium cells.

Vitamin D are essential bioactives that play a very important role in increasing intestinal absorption of calcium, and maintaining the heath of bone, teeth and muscle (Holick 2004b, Gueli *et al.*, 2012). On the other hand, vitamin D deficiency is prevalent in some populations with insufficient exposure of sunlight or poor dietary intake (Nair and Maseeh 2012, Lee *et al.*, 2008). The application of vitamin D in aqueous-based products is problematic due to their poor water-solubility and chemical instability. Therefore, appropriate nanoemulsion/nanodispersion-based delivery systems need to be developed to improve the application of vitamin D in food industries.

1.2 Vitamin D

1.2.1 Historical background of vitamin D

Vitamin D refers to a group of a family of fat-soluble vitamins that commonly exists as two major chemical forms: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) (Fig. 1.1). In fact, it takes long time for scientists and researchers to discover these functional compounds. Rickets, a bone disease related to vitamin D and calcium deficiency, has suffered many peoples worldwide in the past (Weick 1967). The studies of this disease has resulted in the discovery of vitamin D. Daniel Whistler and Francis

Glisson provided scientific descriptions of the disease of rickets in 1645 and 1650, respectively (Glisson *et al.,* 1951). During that time, the risk of rickets occurs quite often for the populations in the United Kingdom, which was known as 'the English Disease' (DeLuca 2014). Edward Mellanby, a famous British doctor, firstly noticed that dogs fed with cod liver oil could be free from the risks of rickets in 1919 (Wolf 2004, Mellanby 1976), although he incorrectly concluded that the 'vitamin A' present in the oil is the main factor responsible for preventing this disease. In 1922, McCollum and his co-workers found that cod liver oil in the absence of vitamin A still has the ability to cure rickets (McCollum *et al.,* 1922). They therefore concluded that another new substance, which was later named as vitamin D, is the true factor that cured rickets. In the meantime, Hess and his group found that and some scientists have noted that the rickets could be cured via sunlight or UV light (Hess *et al.,* 1922). In the early 1920's, Harry Steenbock, a professor at the University of Wisconsin, developed a way to increase vitamin D level in foods via irradiation and then patented it (Weiner 1987). Hess *et al.*, (1925) found that phytosterol or cholesterol became active against rickets after they were irradiated by UV light. They therefore proposed the hypothesis of cholesterol in the skin converted into active vitamin D by sunlight or artificial irradiation. Later, Heilbron *et al.,* (1927) demonstrated that some impurities in the cholesterol (we now know that it is 7-dehydrocholesterol) might be the of vitamin D, rather that cholesterol itself. Windaus and Hess (1926) found that ergocalciferol is the precursor of vitamin D, which is also confirmed by (Rosenheim and Webster 1927). Windaus was award the Nobel Prize in Chemisty in 1928 because of his eccellent work on sterol and their relation to vitamin D. The correct structure of vitmin D_2 was estabilished by Windaus and Thiele (1936). But the question still remained that how human can produce vitamin D via sunlight exposure

because of ergosterol does not exist in human. This mystery remained unsolved untill 1937, when Windans and Bock (1936) isolated and identified a substance from the skin, 7-dehydrocholesterol, which was also present in analmal-based foods (e.g., milk and liver), convertible to an antirachitic substance by irradiation. The irradation product of 7-dehydrocholesterol is vitamin D_3. The estalishment of its strcucture was done by Windaus *et al.*, (1936). The complicate chemical step of converting ergosterol to ergocalciferol was elucidated by Velluz *et al.*, (1955). In 1980, Holick and his team reported the exact sequence of steps required to produce vitamin D_3 via photosynthesis in the skin (Holick *et al.*, 1980). Based on considerable amount of previsous studied, peoples get familiar with vitamin D, as well as the photosynthesis of vitamin D_2 and vitamin D_3, which was shown in Fig. 1.2.

1.2.2 Sources of vitamin D

The most easy and efficient way to obtain vitamin D is exposing the human skin to the sunlight (Holick 2004a). Ultraviolet of B radiation (UVB, wavelength of 290-315 nm) from the sunlight is responsible of converting 7-dehydrocholesterol (pro-vitamin D_3) to previtamin D_3, which quickly converts to vitamin D_3 thermally (Norman and Powell 2005). However, it should be noticed that excessive sunlight exposure could destroy pre-vitamin D_3 and vitamin D_3 by converting them into inactive photoproducts (Holick 2007). In addition, the efficiency of vitamin D_3 production in human skin can be affected by a number of factors, such as time and season, latitude, age or skin color (Holick 2011). For example, people with dark skin need much longer time of sunlight exposure to synthesize the same level of vitamin D_3 than those with white skin, because the dark skin acts as a natural sunscreen to reduce its ability of vitamin D_3 synthesis (Holick 2011). Aging is

also a main factor that decrease the ability of human skin to make vitamin D_3 (MacLaughlin and Holick 1985). Human also can obtained vitamin D from food intakes, however, the sources of vitamin D-contained food is very few. The major dietary sources for vitamin D_3 is fish oil and oily fish such as salmon and mackerel. Beef liver and egg yolk also naturally contain small amount of vitamin D_3. Some fortified foods such as milk, yogurt and cheese also contain the required amount of vitamin D_3. In the case of vitamin D_2, mushrooms are the main dietary sources (Holick 2007). The level of vitamin D_2 in mushrooms is relatively low, but can be largely increased via sun exposure or artificial irradiation. For industrially production, vitamin D_2 is synthesized by UV irradiating the ergosterol from yeast, and Vitamin D_3 obtained by UV irradiating 7-dehydrocholesterol from lanolin (Holick 2007). Table 1.1 listed some sources of vitamin D_2 and vitamin D_3.

1.2.3 Metabolism of vitamin D

Vitamin D from the skin or foods has no biological activity without the metabolic activation occurred in human body. Vitamin D can be stored in and then released from fat cells. Once vitamin D enter to the blood circulation, it would be bound to vitamin D binding protein (DBP), which plays an very important role on transporting vitamin D to liver or kidney for metabolic activation (Holick 2006a). In the liver, vitamin D can be converted into 25-hydroxyvitamin D (25 (OH) D) by the enzymes of 25-hydroxylases (Andersson and Jörnvall 1986, Wikvall 2001, Lund and DeLuca 1966, Holick 2011). 25-hydroxyvitamin D is considered as the circulating form of vitamin D, which is also commonly used for vitamin D status measurement (Seamans and Cashman 2009). 25-hydroxyvitamin D was then transported into kidney, where it is converted into its active form, which is 1,25-dihydroxyvitamin D (1,25 (OH) D), metabolized by the enzyme 25-

hydroxyvitamin D-1α-hydroxylase (CYP27B1) (Fraser and Kodicek 1970, Holick 2007). 25-hydroxyvitamin D goes to the intestine and then interacts with vitamin D receptor (VDR), thereby increasing the calcium absorption. The production of 25-hydroxyvitamin D in the kidney was tightly regulated by the level of plasma parathyroid hormone and serum calcium and phosphorus (Holick 2006b, Bouillon *et al.,* 2001, DeLuca 2004). Fig. 1.3 shows the pathway of vitamin D synthesis and metabolism.

1.2.4 Dietary reference intakes of vitamin D

The required vitamin D intake for individuals depends on a number of factors, including age, race, latitude, and so on. Institute of Medicine (IOM) recommend that 400-800 IU (10-20 μg) of vitamin D daily intake is adequate for 97.5% of the populations (Ross *et al.*, 2011, Heaney *et al.,* 2003). However, daily intake of should be increased for the populations without being exposed to the sun or suffering from vitamin D deficiency. Previous study also suggested that overweight or obese individuals, and postmenopausal women need higher amount of vitamin D intake to reach the desired serum 25 (OH) D concentration, which is around 20-30 ng/ml (Gallagher *et al.,* 2012, Talwar *et al.,* 2007, Ekwaru *et al.,* 2014, Zittermann *et al.,* 2014). Table 1.2 shows the vitamin D dietary reference intakes by life stage.

1.2.5 Toxicity of vitamin D

The toxicity of vitamin D is relatively rare, however, it may occur when huge amount of vitamin D is take from foods or supplements (Pettifor *et al.,* 1995, Blank *et al.,* 1995). Although, the reason for vitamin D toxicity is still in the debate, there are three major hypotheses (Jones 2008): (i) excessive vitamin D intake increase 1,25 (OH) D

concentration, thereby leading to an increase in 1,24 (OH) D concentrations, (ii) excessive vitamin D intake raise the 25(OH) D levels that exceed DBP binding capacity and then directly enter the target cells to bind with VDR, thereby affecting gene expression, (iii) excessive vitamin D intake results in high level of vitamin D metabolites.

Previous studies have pointed out chronic consumption of a high dose of vitamin D (40000 IU per day) could lead to potential risks, such as hypercalcemia and hyperphosphatemia (Vieth 1999, Alshahrani and Aljohani 2013). Hypercalcemia is a result of high level of calcium in the blood serum and the stimulation of bone resorption (Bouillon *et al.*, 2003). The typical symptoms of vitamin D toxicity include lethargy, dehydration, mild nausea, and so on (Blank *et al.*, 1995, Koutkia *et al.*, 2001). IOM suggests that the safe level of serum 25 (OH) D in children and adults is lower than 125-150 nmol/L and 250 nmol/L, respectively. Serum 25 (OH) D concentration of > 375 nmol/L has the potential risk of hypercalcaemia.

1.2.6 Deficiency of vitamin D

IOM defines serum 25 (OH) D concentration of < 20ng/mL (50 nmol/L) as vitamin D deficiency, and serum 25 (OH) D concentration of 21-29 ng/mL (52-73 nmol/L) as vitamin D insufficiency (Holick *et al.*, 2011). As mentioned earlier, rickets and osteomalacia are the major diseases associated with the deficiency of vitamin D. Previous studies suggested vitamin D deficiency also increase the risks of other diseases, including cardiovascular diseases, cancers, immune and inflammatory diseases (Holick 2007). Although, vitamin D_3 can be synthesized in the skin via sunlight exposure, there are still many people who have or at the risk of diseases associated with vitamin D deficiency or insufficiency. Previous work demonstrated that around one billion people worldwide have

vitamin D deficient or insufficient in their blood (Holick 2007). There are many factors resulting in vitamin D deficiency or insufficiency, including environment conditions, lifestyle, and physical characteristics. Fig. 1.4 shows the main reasons contributing to vitamin D deficiency.

1.2.7 Health benefits of vitamin D

As mentioned earlier, the major essential function of vitamin D is to prevent bone-related diseases, such as osteomalacia, which is also know rickets in in children. During the past decades, there are increasing evidences showing that vitamin D plays an important role in a wide range of health, such as improving immune systems, as well as preventing cardiovascular disease and certain types of cancer and a number of other diseases (DeLuca 2004, Holick 2006a, Kulie *et al.,* 2009). The health benefits of vitamin D are summarized in Fig. 1.5.

1.3 Nanoemulsions

1.3.1 Definition

There is an increasing trend of using nanotechnology to produce a wide range of new products in food application due to their special role in modifying the texture, tastes and stability of formed beverages and foods. Nanoemulsions is a system produced from nanotechnology, which commonly have two immiscible liquid phases, with one liquid phase (oil or water phase) dispersed as small droplets within the other (water or oil). Nanoemulsions commonly classified as two forms: oil-in-water (O/W) and water-in-oil (W/O) nanoemulsions, depending on the whether the dispersed droplets is formed from the oil or water phase (Singh *et al.,* 2017). The droplets present in the nanoemulsion

system are typically in the range of 20-200 nm (Aboofazeli 2010), although to some researchers, droplets not exceeding 1 μm (1000 nm) should still be considered to be in the nano-size range (Jaiswal *et al.*, 2015). Nanoemulsions play a very important role in food, pharmaceutical, and chemical applications.

1.3.2 Nanoemulsion formulation

The nanoemulsions can be successfully fabricated by many different methods, but these can usually be divided into high-energy or low-energy approaches depending on the underlying principle (Tadros *et al.*, 2004, McClements and Rao 2011). The formulation of nanoemulsion via high-energy methods requires some mechanical devices, such as high-pressure valve homogenizer, microfluidizers, and ultrasonic generator (Tang *et al.*, 2012, Lee and Norton 2013). All of these devices can generate intense disruptive forces, thereby breaking down larger droplets into smaller ones, until leading to the formulation of nano-sized emulsions (McClements and Rao 2011). The formulation methods, operating setting and system components are the major factors affecting the droplet size formed via high-energy approach (McClements 2011). A recent review article summarized the average droplet size of food-grade nanoemulsions obtained by high-energy methods (Table 1.3) (Acevedo-Fani *et al.*, 2017).

In contrast, the formulation of nanoemulsion by using low-energy methods depends on the changes in physicochemical properties of phases, which leads to the spontaneously formation of nano-sized droplets (McClements and Rao 2011). The most available low-energy methods for producing nanoemulsions are emulsion phase inversion (EPI), spontaneous emulsification (SE), and phase inversion temperature (PIT) (Anton and Vandamme 2009, Ostertag *et al.*, 2012, Gulotta *et al.*, 2014, Hategekimana *et al.*, 2015,

Komaiko and McClements 2016). The detailed mechanisms and factors affecting the droplet formulation via these low-energy methods was clearly explained in recent review papers (Komaiko and McClements 2016, Solans and Solé 2012, McClements and Rao 2011). Both high-energy and low-energy methods have their advantages and disadvantages. For example, low-energy methods are more cost-effective and simple way to produce nanoemeulsions when compared with high-energy methods. However, the low-energy methods commonly require higher surfactant concentrations to reach small droplet size, which could result in toxicity problems in food applications (Öztürk 2017).

1.3.3 Stability of nanoemulsions

Nanoemulsions are thermodynamically unstable due to the relatively lower free energy of separated oil and water phases compared with the emulsified systems (Tadros *et al.*, 2004, McClements 2011). However, the extremely small size of droplet provide nanoemulsions with a highly kinetic stability against (Anton *et al.*, 2008). There are main two mechanisms accounting for the much higher stability of nanoemulsions against gravitational separation, flocculation, and coalescence when compared with conventional emulsions: (i) the velocity of creaming or sedimentation is proportional to the square of the droplet size; (ii) Brownian motion of nano-sized droplets dominates gravitational forces (Tadros *et al.*, 2004). However, the stability of nanoemulsions also depends on the emulsifier type, oil type, environmental conditions, and so one (Wooster *et al.*, 2008, Teo *et al.*, 2016). Ostwald ripening of a nanoemulsion decreases its stability by diffusing the oil phase from small droplets to larger ones (Öztürk 2017). Selection of an appropriate oil phase and the addition of ripening inhibitors can largely slow down the Ostwald ripening to enhance the stability of nanoemeulsions (Wooster *et al.*, 2008, Chang *et al.*, 2012).

1.3.4 Emulsifiers

An emulsifier consists of a hydrophobic tail attached with a hydrophilic head, which plays a crucial important role on formulation and stabilization of nanoemulsion. During homogenization, emulsifiers can quickly absorb onto the new-formed droplets and reduce the interfacial tension between oil-water interfaces to facilitate the formulation of small droplets (Schubert and Engel 2004, Kralova and Sjöblom 2009). Emulsifiers also can act as a barrier to prevent from emulsified droplets from aggregation to provide a long-storage stable emulsion via steric and electrostatic repulsion (Kralova and Sjöblom 2009, Tan *et al.,* 2016c). A variety of emulsifiers that are available for emulsion-based products production, but they may differ in term of physicochemical properties, cost, legal status and the reliability of supply (Hasenhuettl and Hartel 2008, Yang *et al.,* 2013). Each category of emulsifier has its own advantages and disadvantages. In this regard, the selection of emulsifier is an important factor to be considered during the formulation of emulsions. Recently, consumers prefer to choose foods made from natural ingredients. Consequently, there is an increasing trend of using natural emulsifies for producing emulsion-based products. Natural emulsifiers typically contain proteins, polysaccharides, phospholipids, saponins and glycolipids (McClements *et al.,* 2017). In the case of polysaccharides (e.g., gum arabic and pectin), they are difficult to produce small droplets because of their relatively low surface activity, thereby leading to a relatively low physical stability (Ozturk *et al.,* 2015a, Charoen *et al.,* 2011). Some proteins (e.g., gum arabic and pectin) are effective at producing small droplets, however, they were sensitive to be unstable at pH close to their isoelectric point, at high salt concentration, or at high temperature (McClements 2004). Lecithin commonly need to be modified to promote its emulsifying ability via physical, chemical, or enzymatic methods (Weete *et al.,* 1994).

The nanoemulsions prepared by rhamnolipids, which is a typical type of glycolipids, are highly unstable to oil off at low pH and high ionic strength, which strongly limits their application in producing emulsion-based products (Bai and McClements 2016). Recently, a number of previous studies showed saponins (such as quillaja saponin, ginseng saponin, argan saponin, yucca saponin, and so on) are effective in term of preparing nanoemeulsion, however, their stability to salt and acidic pH are problematic (Ralla *et al.,* 2018, Shu *et al.*, 2018, Taarji *et al.*, 2018, Yang *et al.,* 2013). Among of saponin-based emulsifiers, quillaja saponin appears to be the best one due to its relatively higher pH and salt stability than the others. More information regarding the characteristics of natural emulsifiers can be obtained from two perfect review publications (McClements *et al.*, 2017, Ozturk and McClements 2016).

1.4 Objectives and structure of the thesis

Considering the aforementioned a large number of populations worldwide whom are or at risks of vitamin D insufficiency, it is necessary to develop foods and beverages incorporating this vitamin. However, the utilization of vitamin D has big challenges due to its low water-solubility, poor chemical stability against light, oxygen, or elevated temperatures, as well as the low bioavailability. One strategy is in using nanoemulsion/nanodispersion-based delivery systems to overcome these limitations.

The work presented herein mainly focus on the development and characterization of ergocalciferol-loaded nanoemulsion/nanodispersions stabilized by different emulsifiers. The objectives of the study in this dissertation are listed as follow:

I. To prepare nano-sized nanoemulsion/nanodispersions loaded with ergocalciferol using different emulsifiers via high-energy or low-energy methods.

II. To study the factors affecting the formulation, stability, and bioaccessibility of nanoemulsion/nanodispersions encapsulating ergocalciferol.

III. To elucidate the mechanisms of formulation, stability, and bioaccessibility of nanoemulsion/nanodispersions encapsulating ergocalciferol.

The structure of the thesis was showed in Fig. 1.6. In chapter 1, a general introduction of vitamin D and nanoemulsion/nanodispersion was reviewed. In the chapter 2, we focus on the formulation of nanoemulsions loaded with ergocalciferol via high-pressure homogenization method, in which some important factors (such as emulsifier type and concentration, oil type and concentration, and homogenization pressure) affecting the droplet size and size distribution were investigated. In the chapter 3, the effect of emulsifier type on the stability of nanoemulsions loaded with ergocalciferol was investigated. In chapter 4, the effect of emulsifier type on the droplet characteristics, lipid digestion, and ergocalciferol bioaccessibility of nanoemulsions investigated via an in vitro digestion model. In chapter 5, ergocalciferol nanodispersions were prepared by low-energy method. The effect of emulsifier type on the formulation, stability and bioaccessibility was studied. In chapter 6, a general conclusion from the present work was summarized.

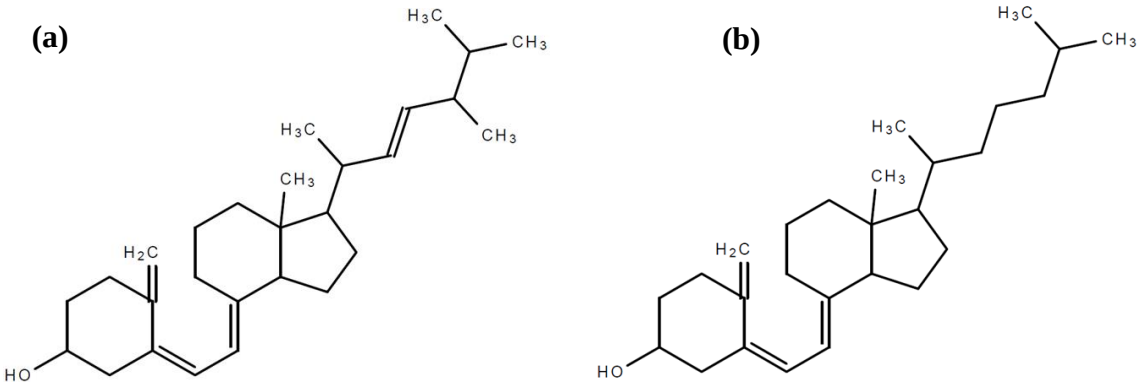


Fig. 1. 1: Chemical structures of vitamin D. (a) Vitamin D_2 and (b) Vitamin D_3.

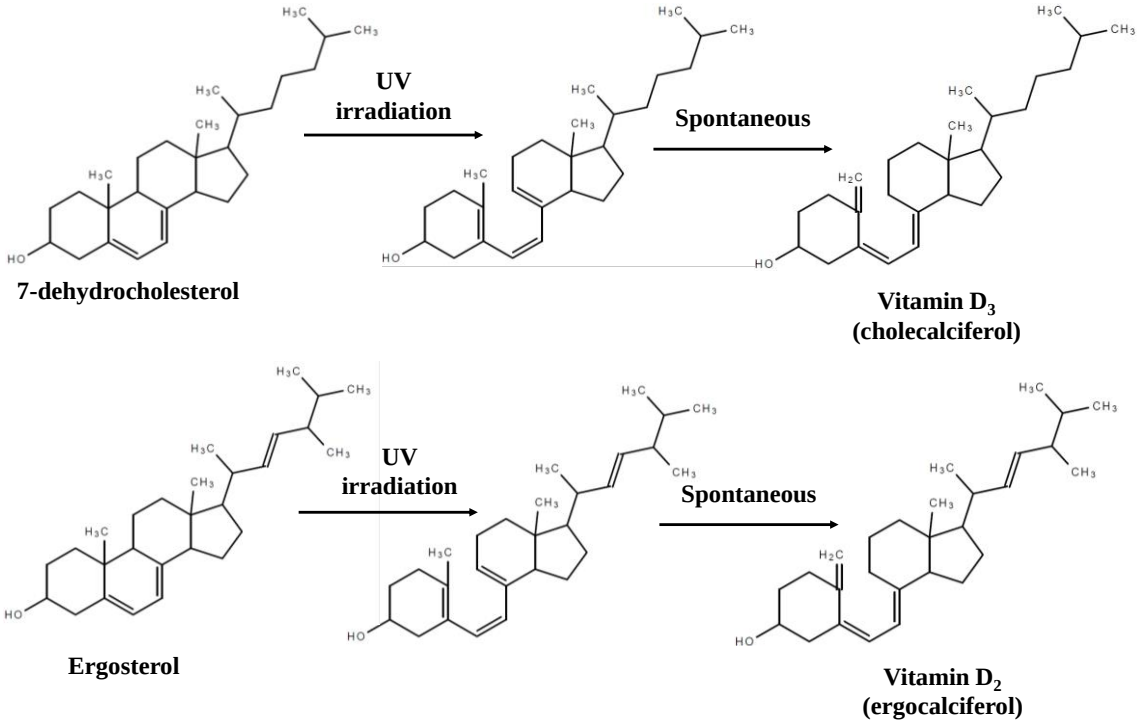


Fig. 1. 2: Photochemical reaction steps from pro-vitamin D (7-dehydrocholesterol or ergocalciferol) to vitamin D (cholecalciferol or ergocalciferol).

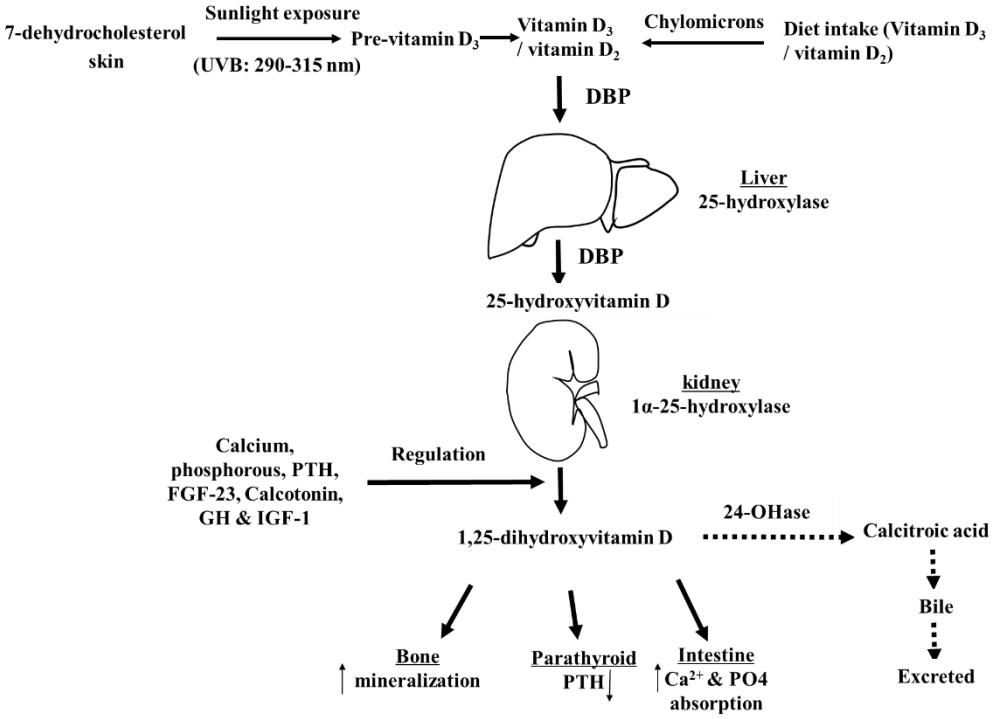


Fig. 1. 3: Vitamin D metabolism and classical actions on mineral metabolism. Modified from (Holick 2007) and (El fakhri 2016). DBP: vitamin D binding protein; PTH: parathyroid hormone; FGF-23: fibroblast growth factor-23; GH: growth hormone; IGF-1: insulin-like growth factor-1.

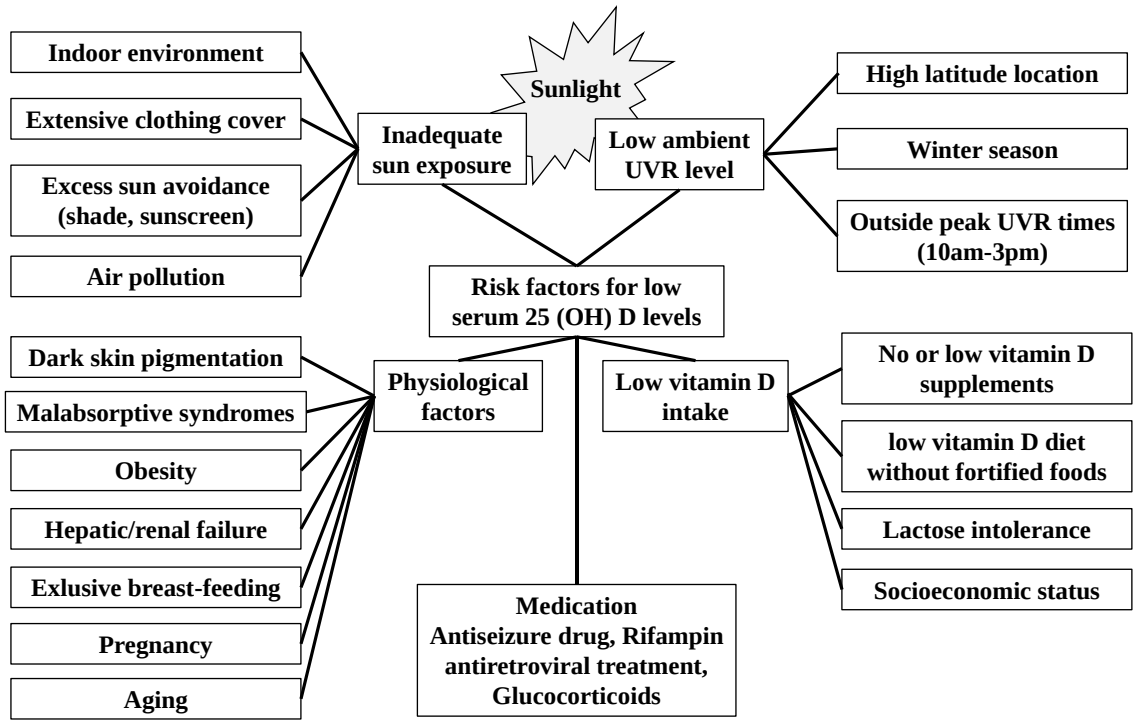


Fig. 1. 4: Causes of vitamin D deficiency. Source: (Hossein-nezhad and Holick 2012).

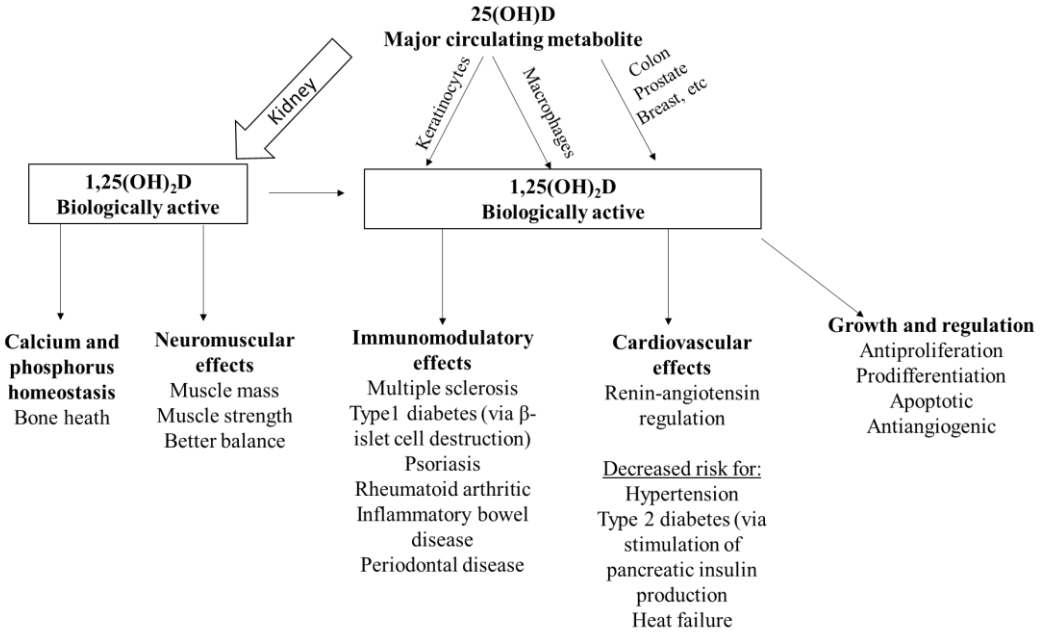


Fig. 1. 5: Health benefits of vitamin D, adapted from (Holick 2006a)

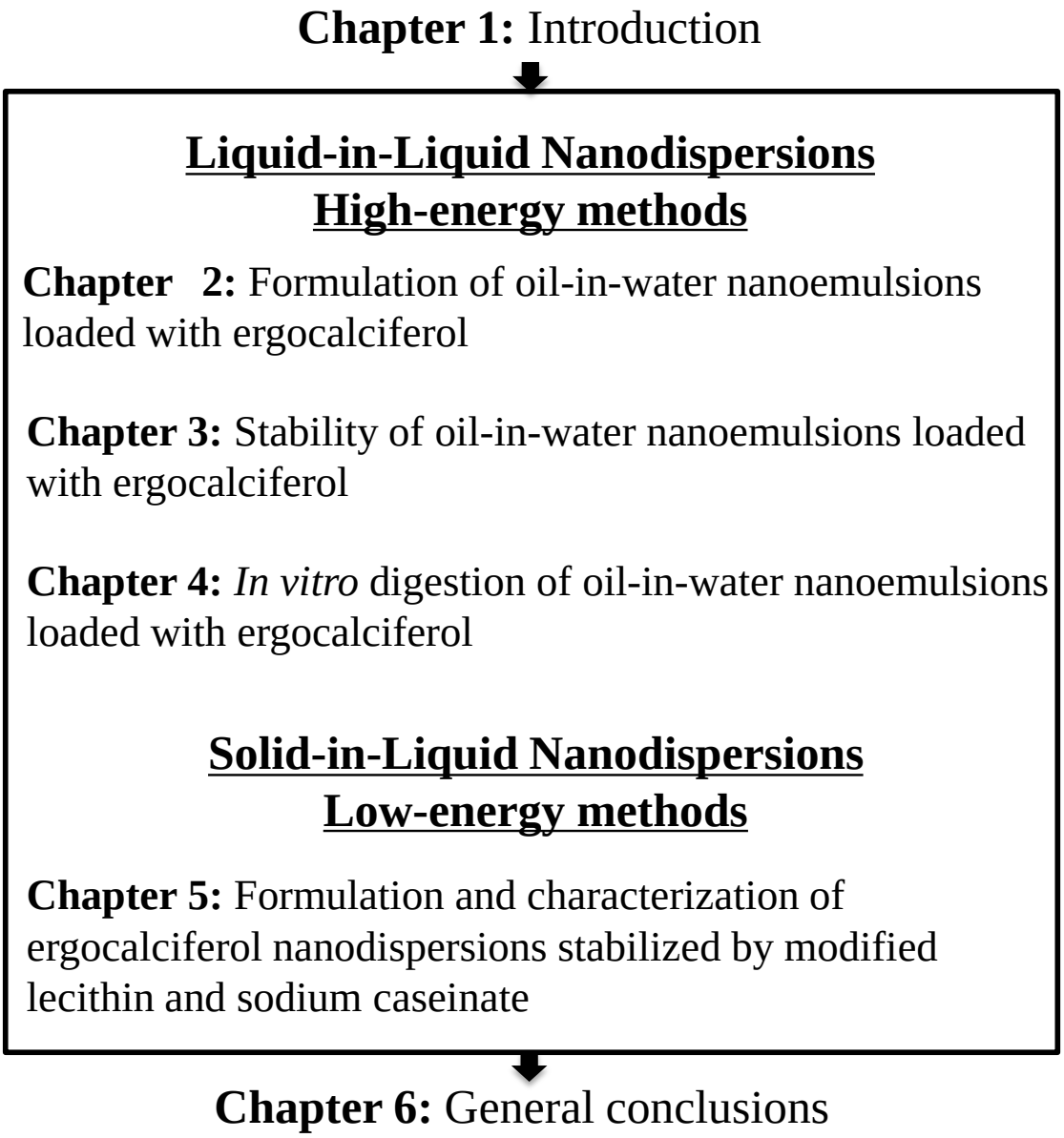


Fig. 1. 6: General structure of this thesis.

Table 1. 1: Sources of Vitamin D_2 and Vitamin D_3. Source: (Holick 2007) and (Holick 2011).

Sources	Vitamin D content (IU = 25 ng)
Natural sources	
Cod liver oil	~400-1000 IU/tsp vitamin D_3
Salmon, fresh wild caught	~600-1000 IU/3.5 oz vitamin D_3
Salmon, fresh farmed	~100-250 IU/3.5 oz vitamin D_3, vitamin D_2
Salmon, canned	~300-600 IU/3.5 oz vitamin D_3
Sardines, canned	~300 IU/3.5 oz vitamin D_3
Mackerel, canned	~250 IU/3.5 oz vitamin D_3
Tuna, canned	236 IU/3.5 oz vitamin D_3
Shiitake mushrooms, fresh	~100 IU/3.5 oz vitamin D_2
Shiitake mushrooms, sun dried	~1,600 IU/3.5 oz vitamin D_2
Egg yolk	~20 IU/yolk vitamin D_3 or D_2
Sunlight/UVB radiation	~20,000 IU equivalent to exposure to 1 minimal erythemal dose (MED) in a bathing suit. Thus, exposure of arms and legs to 0.5 MED is equivalent to ingesting ~ 3,000 IU vitamin D_3.
Fortified foods	
Fortified milk	~100 IU/8 oz usually vitamin D_3
Fortified orange juice	~100 IU/8 oz vitamin D_3
Infant formulas	~100 IU/8 oz vitamin D_3
Fortified yogurts	~100 IU/8 oz usually vitamin D_3
Fortified butter	~56 IU/3.5 oz usually vitamin D_3
Fortified margarine	~429IU/3.5 oz usually vitamin D_3
Fortified cheeses	~100 IU/3 oz usually vitamin D_3
Fortified breakfast cereals	~100 IU/serving usually vitamin D_3
Supplements	
Multivitamin	400, 500, 1000 IU vitamin D_3 or vitamin D_2
Vitamin D_3	400, 800, 1000, 2000, 5,000, 10,000, and 50,000 IU

Table 1. 2: Vitamin D dietary reference intakes by life stage (amount/day). AI: adequate intake; EAR: estimated average requirement; RDA: recommended dietary allowance; UL: tolerable upper intake level; IU = 25ng. Source: (Del Valle *et al.*, 2011).

Life Stage Group	AI	EAR	RDA	UL
Infants				
0-6 months	400 IU			1000 IU
6-12 months	400 IU			1500 IU
Children				
1-3 years		400 IU	600 IU	2500 IU
4-8 years		400 IU	600 IU	3000 IU
Males				
9-13 years		400 IU	600 IU	4000 IU
14-18 years		400 IU	600 IU	4000 IU
19-30 years		400 IU	600 IU	4000 IU
31-50 years		400 IU	600 IU	4000 IU
50-70 years		400 IU	600 IU	4000 IU
>70 years		400 IU	800 IU	4000 IU
Females				
9-13 years		400 IU	600 IU	4000 IU
14-18 years		400 IU	600 IU	4000 IU
19-30 years		400 IU	600 IU	4000 IU
31-50 years		400 IU	600 IU	4000 IU
51-70 years		400 IU	600 IU	4000 IU
>70 years		400 IU	800 IU	4000 IU
Pregnancy				
14-18 years		400 IU	600 IU	4000 IU
19-30 years		400 IU	600 IU	4000 IU
31-50 years		400 IU	600 IU	4000 IU
Lactation				
14-18 years		400 IU	600 IU	4000 IU
19-30 years		400 IU	600 IU	4000 IU
31-50 years		400 IU	600 IU	4000 IU

Table 1. 3: Recent studies in active edible coatings containing naturally origin food substances. Source: (Acevedo-Fani *et al.*, 2017).

Methods	**Conditions**	**Composition**	**Droplet size (nm)**
High pressure homogenization	Pressure: 50, 100, and 150 MPa Number of passes: 1–20	Clove oil in canola oil, succinylated waxy maize starch solutions	150–400
	Pressure: 20 000 psi Number of cycles: 6	Canola oil, sodium caseinate, pea protein concentrate solutions	≈160
Microfluidization	Pressure: 34.5–206.8 MPa Number of passes: 1–5	Mesquite gum solutions and fish oil	155–300
	Pressure: 150 MPa Number of passes: 5	High methoxyl pectin solutions, Tween 80 and essential oils	12–40
	Pressure: 150 MPa Number of cycles: 5	Sodium alginate, Tween 20 and corn oil	≈260
Sonication	Amplitude 80 μm, cycle 0.7 and 20 min	Whey protein isolate solutions and fish oil	66–703
	20.5 kHz for 15 min	Sage oil, Tween 80, Span 80 and water	222
	24 kHz for 1–20 min	Flaxseed oil, algae oil, Tween 40, lecithin and water	182–192

Chapter 2

Formulation of Oil-in-Water Nanoemulsions Loaded with Ergocalciferol

2.1 Introduction

Vitamin D deficiency or insufficiency is prevalent in some populations, which lead to adverse health effects, such as rickets. Ergocalciferol, one major form of vitamin D, plays an important role in enhancing intestinal calcium absorption, and reducing the risks of rickets and bone-related diseases (Holick 2004b, Gueli *et al.*, 2012). Thus, the foods and beverages containing vitamin D are preferred by consumers. However, there are a number of challenges for using vitamin D as micronutrients in food industries, which is due to its poor water-solubility and chemical stability, as well as low bioaccessibility (Luo *et al.*, 2012, Ozturk *et al.,* 2015b).

Oil-in-water (O/W) nanoemulsions are excellent candidates for effectively delivering those functional lipophilic components due to their important role in increasing water-solubility and bioaccessibility (Salvia-Trujillo *et al.*, 2015, Zhang *et al.,* 2016, Ahmed *et al.*, 2012, Yang *et al.,* 2017). In the current studies, we aimed to formulate ergocalciferol-loaded nanoemulsion by using high-pressure homogenization method. A various factors affecting on the formulation of nanoemulsions were investigated, including emulsifier type and concentration, oil content and type, and homogenization pressure.

2.2 Materials and methods

2.2.1 Materials

Ergocalciferol, soybean oil, D-limonene, and sodium caseinate (SC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Medium chain triglycerides (MCT-7) were purchased from Taiyo Kagaku Co., Ltd. (Mie, Japan). Perilla oil was purchased from a local supermarket in Japan. Decaglycerol monooleate (MO7S) was provided by Sakamoto Yakuhin Kogyo Co., Ltd. (Osaka, Japan). SC used in our study has a maximum

loss of 10% after drying and its total nitrogen (N) is 12.6-15.8 %.MO7S (polymerization degree 10), which has a hydrophilic-lypophilic balance (HLB) value of 12.9. The modified lecithin (ML; SLP WhiteLyso), containing phosphatidic acid (0-5%), phosphatidylethanolamine (1-7%), phosphatidylcholine (2-8%), phosphatidylinositol (10-20%) and lysophosphatidylcholine (18-30%), was purchased from Tsuji Oil Mills Co., Ltd. (Tokyo, Japan). ML has a HLB value around 12. Milli-Q water (18 MΩ cm) was used to prepare all of the solutions and samples. All other chemicals used were of analytical grade.

2.2.2 Formulation of ergocalciferol-loaded nanoemulsions

O/W nanoemulsion loaded with ergocalciferol were produced using a two-step homogenization method. A series of different oil (either soybean oil, MCT, perilla oil, or D-limonene) containing 0.1 wt% of ergocalciferol were used as oil phases. Phosphate buffer (5 mM, pH7) containing an emulsifier (0.1-3 wt% of ML, MO7S, or SC) was used as aqueous phases. In the first step, premixed emulsions were prepared by homogenizing oil phases with aqueous phases at an appropriate ratio via a conventional homogenizer (Polytron PT-3100, Kinematica Co., Ltd., Luzern, Switzerland) at 5000 rpm for 5 min. In the second step, the premixed emulsions were treated using a high-pressure homogenizer (NanoVater200, Yoshida Kikai Co., Ltd., Nagoya, Japan) at various pressures (20-140 Mpa) for 4 passes to obtain fine emulsions.

2.2.3 Measurements of droplet size and size distribution

The droplet size and size distribution of the resulting emulsion samples were measured using a laser diffraction particle size analyzer (LS 13 320, Beckman Coulter, Brea, USA).

The refractive indexes of water, perilla oil, soybean oil and MCT and D-limonene were set at 1.333, 1.476, 1.471, 1.450 and 1.471, respectively. The average droplet diameter of each sample was reported as volume mean diameter ($d_{4,3}$): $d_{4,3} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$. Where n_i is the number of droplet i; d_i is the diameter of the droplet i. All measurements were conducted in triplicate.

2.2.4 Statistical analysis

All experiments were repeated at least in duplicate and standard deviations were calculated from these measurements. Analysis of variance (ANOVA) tests were used to analyze the characterization and stability data at a confidence level of 95%. Least significant difference (LSD) was used to compare the stability data with different emulsifiers. The LSD was calculated using Statistix 8.1 software (Tallahassee, USA) and according to the method described by Steel and Torrie (2007).

2.3 Results and discussion

2.3.1 Effect of emulsifier concentration on the formulation of nanoemulsions

We initially investigated the influence of emulsifier concentration on the formulation of ergocalciferol-loaded nanoemulsions. A series of nanoemulsions were prepared by homogenizing 10 wt% of soybean oil containing ergocalciferol with 90 wt% of aqueous phases containing 0.1-3 wt% of ML at a constant homogenization condition (100 MPa, 4 passes). In general, the average droplet size ($d_{4,3}$) of ML-stabilized nanoemulsions decreased with increasing emulsifier concentration (Fig. 2.1a). For example, nanoemulsions stabilized by 0.1 wt% of ML had $d_{4,3}$ of around 340 nm, which was much bigger than that stabilized by 2 wt% of ML, which was around 160 nm. Evident broader

droplet size distribution with large droplets in nanoemulsions stabilized by higher ML concertation, which was indicated in Fig. 2.1b. The decreased $d_{4,3}$ of emulsions with increasing ML concentration was relate to the fact that high level of emulsifier molecules present could quickly and effectively adsorb onto the newly-formed droplets, thereby promoting the formulation of small droplets during homogenization (Jafari *et al.*, 2008).

2.3.2 Effect of homogenization pressure on the formulation of nanoemulsions

The impact of homogenization pressure on the formulation of ergocalciferol-loaded nanoemulsions was showed in Fig. 2.2. In this part, the nanoemulsions were prepared by homogenizing 10 wt% of soybean oil containing ergocalciferol with 90 wt% of aqueous phases containing 2 wt% of ML at homogenization pressure of 20-140 MPa. The $d_{4,3}$ of ML-stabilized nanoemulsions decreased from around 470 nm to 145 nm when the operating pressure was increased from 20 to 140 MPa (Fig. 2.2a). Results of droplet size distribution also indicated that the droplets are bigger and broader at the lower homogenization pressure applied (Fig. 2.2b). This effect was due to an increase in disruptive energy generated at high homogenization pressure, thereby resulting in formulation of smaller droplets (Schultz *et al.*, 2004).

2.3.3 Effect of oil content on the formulation of nanoemulsions

The impact of oil mass fraction on the formulation of ergocalciferol-loaded nanoemulsions was showed in Fig. 2.3. In this section, the nanoemulsions were prepared by homogenizing 10-30 wt% of soybean oil containing ergocalciferol with 90-70 wt% of aqueous phases containing 2 wt% of ML at homogenization pressure of 100 MPa. The droplet size and size distribution measurements indicated that nanoemulsions with higher

oil mass fraction had bigger $d_{4,3}$ compared with those with lower oil mass fraction (Figs. 2.3a and 2.3b). The bigger size of high-lipid emulsion could be attributed to that the emulsifier molecules present might be insufficient to cover all newly-formed droplets because of the larger surface area, which was similar to that described in Section 2.3.1.

2.3.4 Effect of oil type on the formulation of nanoemulsions

The impact of oil type on the formulation of ergocalciferol-loaded nanoemulsions was showed in Fig. 2.4. In this section, the nanoemulsions were prepared by homogenizing 10 wt% of different oil (either soybean oil, MCT, perilla oil or D-limonene) containing ergocalciferol with 90 wt% of aqueous phases containing 2 wt% of ML at homogenization pressure of 100 MPa. The droplet size and size distribution measurements indicated that nanoemulsions prepared from D-limonene had much bigger $d_{4,3}$ compared with the other three emulsions (Figs. 2.4a and 2.4b). The observed larger droplets in D-limonene emulsions could be relate to its relatively higher water-solubility, which make them more susceptible to Ostwald ripening (Rao and McClements 2012)

2.3.5 Effect of emulsifier type on the formulation of nanoemulsions

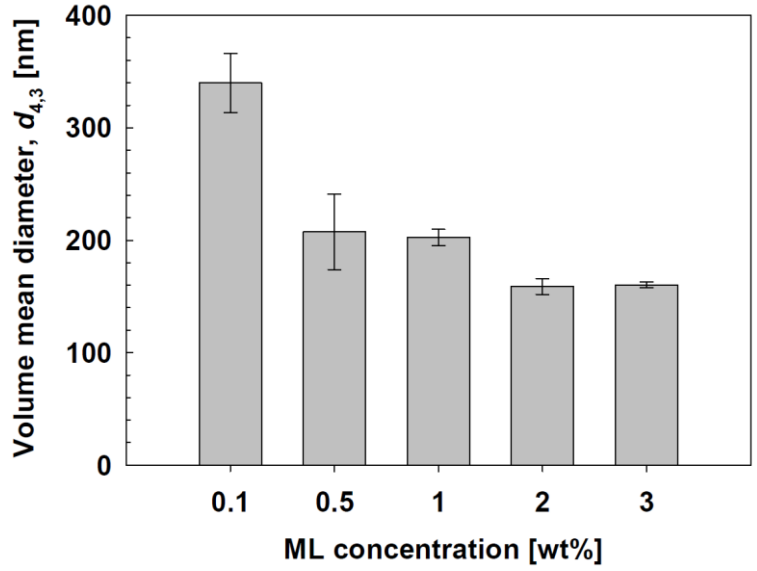
The impact of emulsifier type on the formulation of ergocalciferol-loaded nanoemulsions was showed in Fig. 2.5. In this section, the nanoemulsions were prepared by homogenizing 10 wt% of soybean oil containing ergocalciferol with 90 wt% of aqueous phases containing 2 wt% of different emulsifier (either ML, MO7S or SC) at homogenization pressure of 100 MPa. The three nanoemulsions showed similar mean droplet size and size distribution, regardless of emulsifier type (Figs. 2.5a and 2.5b). As previous study reported (Mao *et al.*, 2009b), small-molecule surfactants can absorb onto

the droplet surface more rapidly than emulsifier with large molecule such as protein, hence leading to form smaller droplet size. Interesting, bigger $d_{4,3}$ of SC-stablized nanoemulsions comparing those stabilized by ML and MO7S was not observed in our study. This phenomenon can be explained by relatively high concentration of SC used in our study can provide sufficient emulsifier molecular to cover the newly formed droplets to prevent them from re-aggregation with each other during high pressure homogenization process, hence be capable of producing droplets with small size (Kanafusa *et al.*, 2007).

2.4 Conclusions

The results in this study demonstrated that nanoemulsions loaded with ergocalciferol could be successfully formulated via high-pressure homogenization method, but highly depend on the emulsifier concentration, oil type and concentration, as well as homogenization pressure. The droplet size of the resulting nanoemulsions decreased with increasing operating pressure and emulsifier concentration. The nanoemulsions prepared from soybean oil, MCT or perilla oil had similar droplet size, and much smaller than that from D-limonene. It was also found that ML, MO7S and SC led to similar and small droplets of nanoemulsions prepared at the same condition.

(a)



(b)

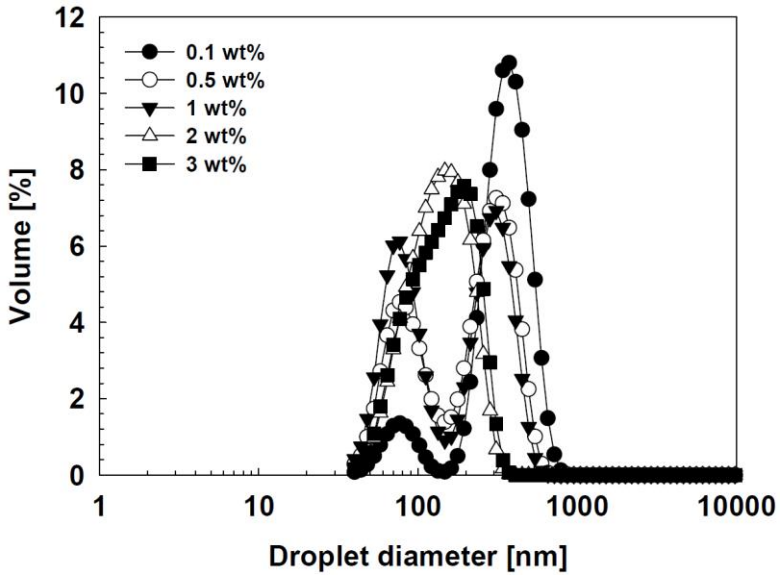
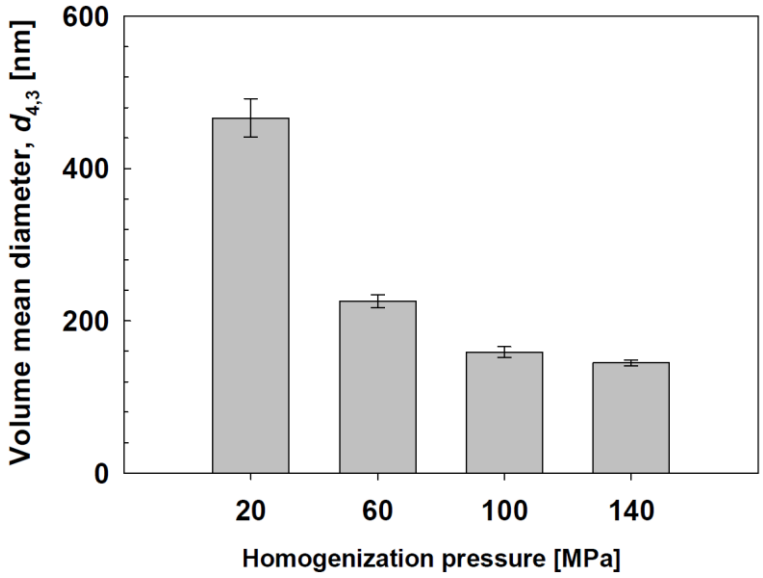


Fig. 2. 1: Effect of ML concentration on (a) mean droplet size ($d_{4,3}$) and (b) size distribution of ergocalciferol-loaded nanoemulsions prepared at a constant emulsification condition (10 wt% soybean oil; 100 MPa, 4 passes).

(a)



(b)

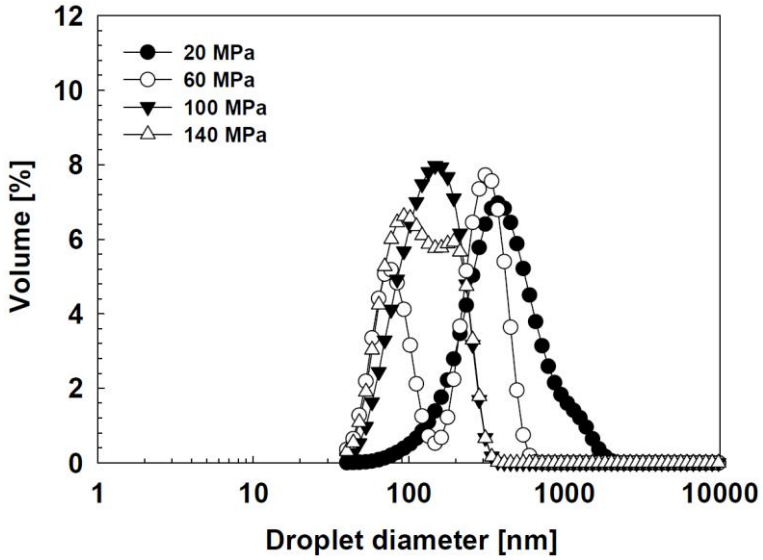
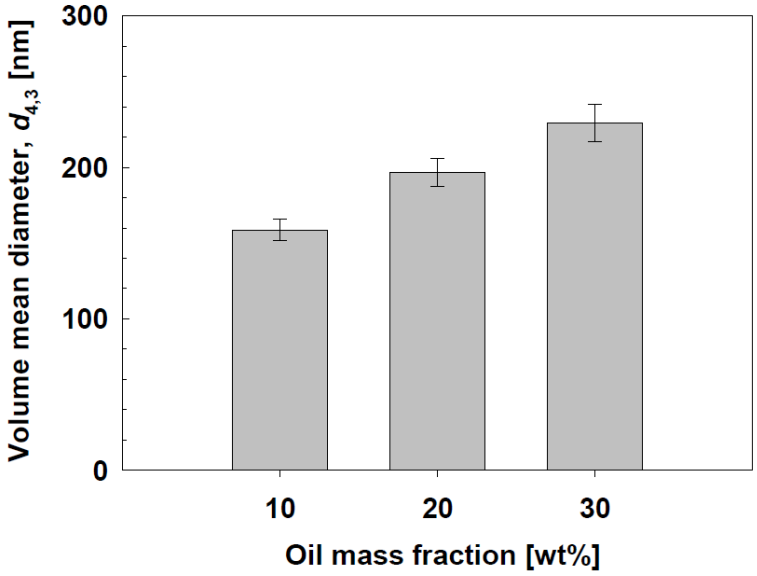


Fig. 2. 2: Effect of homogenization pressure on (a) mean droplet size ($d_{4,3}$) and (b) size distribution of ergocalciferol-loaded nanoemulsions (2 wt% ML ; 10 wt% soybean oil).

(a)



(b)

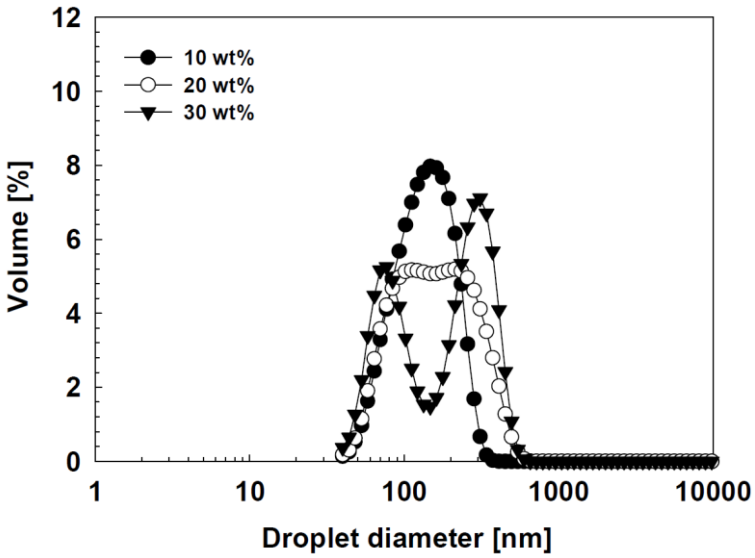
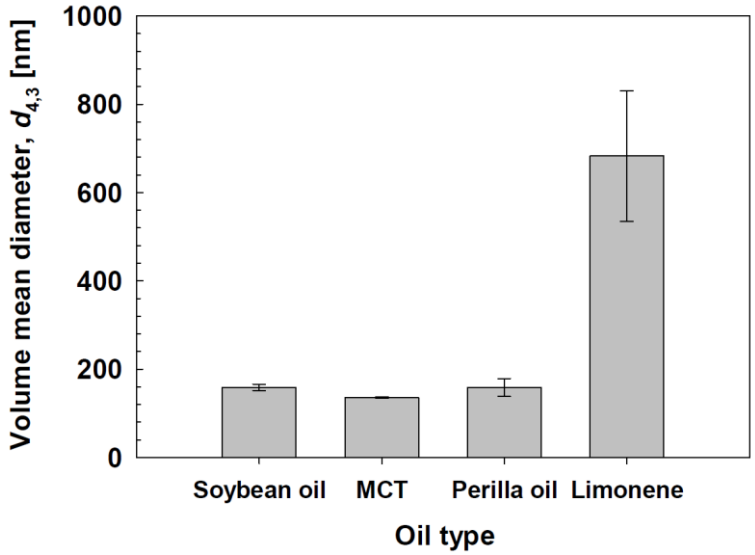


Fig. 2. 3: Effect of soybean oil mass fraction on (a) mean droplet size ($d_{4,3}$) and (b) size distribution of ergocalciferol-loaded nanoemulsions stabilized by 2 wt% ML (100 MPa, 4 passes).

(a)



(b)

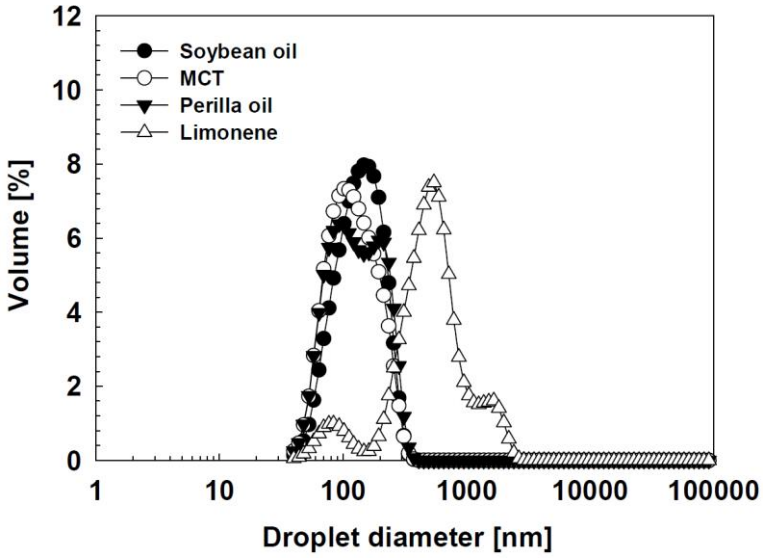
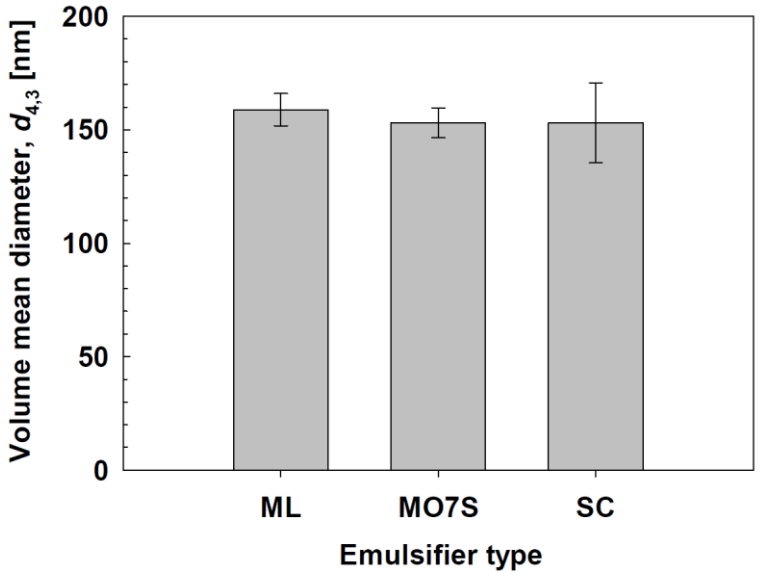


Fig. 2. 4: Effect of oil type (10 wt%) on (a) mean droplet size ($d_{4,3}$) and (b) size distribution of ergocalciferol-loaded nanoemulsions stabilized by 2 wt% ML (100 MPa, 4 passes).

(a)



(b)

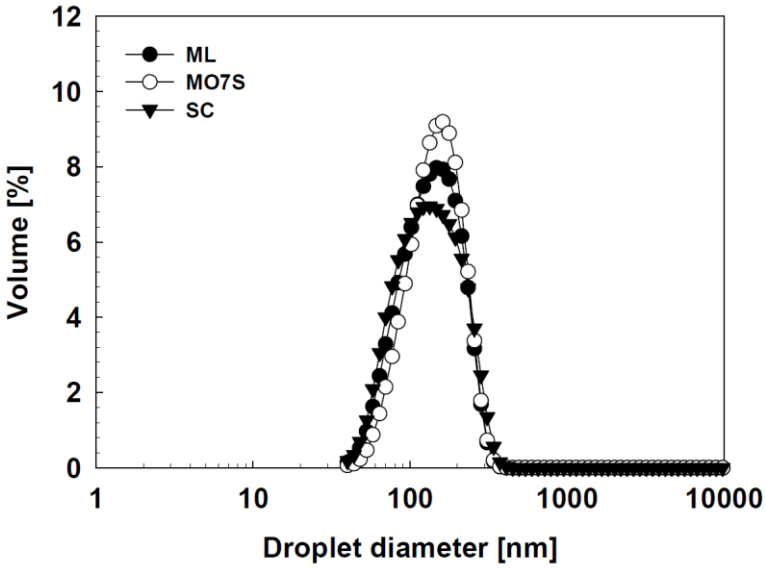


Fig. 2. 5: Effect of emulsifier type (2 wt%) on (a) mean droplet size ($d_{4,3}$) and (b) size distribution of ergocalciferol-loaded nanoemulsions (soybean oil; 100 MPa, 4 passes).

Chapter 3

Stability of Oil-in-Water Nanoemulsions Loaded with Ergocalciferol

3.1 Introduction

Recently, food and beverage products consisting of functional ingredients with additional health benefits attract an increasing attention in food industries (Piorkowski and McClements 2014). Vitamin D is one of the most important nutraceutical compound gaining attention from researchers and food manufacturers due to its special role in maintaining the bone, teeth and cartilage development (Cranney *et al.*, 2008, Hark and Deen 2005). In addition, vitamin D provides prevention against heart diseases, cancer and immune diseases (Haham *et al.*, 2012, Holick 2004c). Vitamin D is a seco-steroid hormone that can be classified into two main different forms, namely vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_2 is naturally present in mushrooms with low amount and commercially produced by UV irradiation of yeast, while vitamin D_3 is naturally synthesized in the skin of human and animal bodies via the exposure to sunlight (Khalid *et al.*, 2015). However, an estimated one billion people worldwide have either vitamin D deficiency or insufficiency (Holick 2007). The deficiency of vitamin D can be ascribed to the lack of exposure to sunlight, extensive use of UV protecting sun cream, or poor intake of food containing vitamin D (Haham *et al.*, 2012, Holick 2004c, Tsiaras and Weinstock 2011). Consequently, food and beverage incorporated this vitamin have raised considerable interest in practical applications. The utilization of vitamin D as nutraceutical ingredient in processed foods or beverages still represents a big challenge for several reasons: vitamin D is a highly hydrophobic compound that cannot be directly dispersed in aqueous phase; chemical degradation of vitamin D occurs when exposed to light, oxygen, or elevated temperatures, thus leading to the reduction of functionality and bioavailability (Guttoff *et al.*, 2015, Tsiaras and Weinstock 2011).

In the past few decades, nanotechnology has received an increasing attention in foods, cosmetics and pharmaceutical applications, and it plays a very important role in encapsulating poor water-soluble bioactive compounds, *i.e*. polyunsaturated fatty acids, carotenoids, phytosterols, and many other ingredients. Several attempts were also carried out to improve the water solubility, stability and bioavailability of vitamin D using different nano-delivery systems including nanoparticles, solid lipid nanoparticles (SLN), nanoliposomes and so on (Abbasi *et al.*, 2014, Patel *et al.*, 2012, Guttoff *et al.*, 2015, Ozturk *et al.*, 2015c, Mohammadi *et al.*, 2014). O/W nanoemulsions is one of the most important nano-delivery systems for encapsulating lipophilic compounds. The nanoemulsions are the colloidal dispersions containing small lipid droplets (typically around 20-200 nm) dispersed with in aqueous phase (Mohammadi *et al.*, 2014, McClements 2015, McClements 2010). Nanoemulsions have high optical clarity, increased oral bioavailability and great stability against gravitational separation and droplet coalescence (McClements 2010, Guttoff *et al.*, 2015, Zhao *et al.*, 2013, Tadros *et al.*, 2004). Keeping these advantages in mind, the nanoemulsions are expected to be useful for encapsulating lipophilic compounds, such as oil-soluble vitamins and nutraceuticals, for application in food and beverage products.

Emulsion-based foods need to be stable against harsh condition (*i.e*., pH, ionic strength and temperature) during their processing, storage and transportation. It is well known that an emulsifier is one of the most important materials required to determine the stability of an emulsion-based food against the destabilization process. The selection of appropriate emulsifier is critical for producing emulsion-based products with high stability (Schubert and Engel 2004). In the current study, three food-grade emulsifiers from different group, namely, modified lecithin (ML), sodium caseinate (SC) and decaglycerol monooleate

(MO7S) were used for evaluating their effect on the physicochemical stability of vitamin D-loaded nanoemulsions. The three emulsifiers were selected due to fact that they provide different stabilizing mechanisms to protect emulsion droplets from aggression. Limited systematic study was available on the comparison of influence of emulsifier with different stabilizing mechanisms on the stability of nanoemulsions loaded with vitamin D. ML used in our work is an enzymatically modified phospholipid derived from hydrolysis of soy lecithin. ML is a mixture of different phospholipids, in which lysophosphatidylcholine is the major compound and phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid are the secondary compounds (Sono 2005). ML is a zwitter-ionic surfactant with small molecular weight, which stabilizes nanoemulsions by electrostatic repulsion. SC is a large molecular weight emulsifier derived from bovine milk, and it can provide a thick coating layer around the oil droplet to prevent droplet from growth mostly due to electrostatic repulsion. In addition, the long tail of disordered casein molecules adsorbed around the oil droplet provides partly steric stabilization effect (Raikos 2010). MO7S, a type of polyglycerol esters of fatty acids (PGEs), is synthetic non-ionic surfactant with small molecular size, which rapidly adsorbs onto oil droplet during homogenization and stabilizes the nanoemulsions by steric hindrance. The objective of this work was to provide a better understanding of the role of emulsifiers with different stabilizing mechanisms on the stability of oil-in-water nanoemulsions encapsulated ergocalciferol. The results obtained from this study provide useful information for the utilization of emulsifiers in the development of nanoemulsion-based delivery system for lipophilic bioactive compounds.

3.2 Materials and methods

3.2.1 Materials

Ergocalciferol, refined soybean oil, sodium caseinate, hydrochloric acid, sodium azide, ethanol (99.8%), sodium hydroxide, D (+)-Glucose, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Modified lecithin (ML, SLP White Lyso) was procured from Tsuji Oil Mills Co. Ltd. (Tokyo, Japan). Decaglycerol monooleate (MO7S) was kindly donated by Sakamoto Yakuhin Kogyo Co. (Osaka, Japan). Milli-Q water with a resistivity of 18 MΩ cm was used for preparing all the solution in the current study. All other chemicals used in this study were of analytical grade and used as such during experimentation.

3.2.2 Preparation of ergocalciferol-loaded O/W nanoemulsions

Ergocalciferol nanoemulsions containing 90 wt% of aqueous phase (buffer solution with 2 wt% ML, SC or MO7S) and 10 wt% of oil phase (soybean oil with 0.1 wt% ergocalciferol) were formulated by high-pressure homogenization method (100 MPa, 4 Passes), which was described in Section 2.2.2 (Chapter 2).

3.2.3 Emulsion stability testing

3.2.3.1 Effect of pH on the stability of nanoemulsions

The pH of ergocalciferol loaded nanoemulsions was adjusted within the range between 2 and 8 using 1 M HCl or 1 M NaOH. Samples were kept at room temperature for 24 h prior to droplet size and ζ-potential measurements.

3.2.3.2 Effect of ionic strength on the stability of nanoemulsions

5 mL of ergocalciferol loaded nanoemulsions were diluted by adding 5 mL NaCl solution (in phosphate buffer, pH 7) to adjust the NaCl concentration in samples (0-500 mM). The samples were stored at room temperature (25 ± 2 ^{o}C) for 24 h prior to analysis.

3.2.3.3 Effect of freeze-thawing on the stability of nanoemulsions

In this section, the influence of freeze-thaw cycles on the stability of ergocalciferol loaded nanoemulsions was evaluated in the absence and presence of glucose as cryoprotectant. Nanoemulsions with additives (emulsions: 10% glucose in phosphate buffer solution (pH 7) =1:1 (v/v)) and without additive (emulsions and phosphate buffer =1:1) were transferred to separate plastic test tubes. The test samples were placed at -20 ^{o}C for 22 h, and then left to thaw in a water bath at 30 ^{o}C for 2 h prior to droplet size measurement.

3.2.3.4 Effect of high temperature treatment on the stability of nanoemulsions

10 mL nanoemulsions encapsulating ergocalciferol were shifted to a 15 mL glass test tube and well-sealed by a metallic cap. The samples were placed in oil bath or autoclaving device (KTS-2346, ALP Co., Ltd, Tokyo, Japan) at fixed temperature (80, 100 or 120 ^{o}C). After heating for 1h, the samples were cooled at room temperature for 1 day prior to analysis.

3.2.3.5 Storage stability of nanoemulsions

The ergocalciferol loaded nanoemulsions with the addition of sodium azide (0.02 wt%) as antibacterial agent were incubated at 25 ± 2 ^{o}C and 55 ± 2 ^{o}C for 30 days under dark condition. The droplet size and ergocalciferol content retained in emulsion were measured

under different storage intervals for a period of 30 days. Ergocalciferol encapsulation efficiency (EE) in samples was calculated with the following equation:

$$EE = \frac{C_t}{C_0} \times 100 \qquad (3.1)$$

where C_t is the concentration of ergocalciferol at a specific time, while C_0 is the initial concentration of ergocalciferol loaded in the nanoemulsions.

3.2.4 Droplet size analysis

The measurement of droplet size was conducted according to the presented method in Section 2.2.3 (Chapter 2)

3.2.5 ζ-potential measurement

Determination of the ζ-potential of nanoemulsions droplet was performed by using a ζ-potential analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). Before analysis, the emulsions at different pH were diluted (1: 200) with Milli-Q water at the same pH as the initial samples being tested. The emulsions at different salt concentrations were diluted (1: 100) with phosphate buffer (5 mM, pH 7). The diluted samples were then placed in a folded capillary cell and loaded in the instrument. The ζ-potential of samples were automatically measured using 10-100 runs per analysis after they were equilibrated for 120 s inside the instrument at 25 °C. The refractive indexes of the dispersed and continuous phases for calculation were set at 1.471 and 1.333, respectively, each measurement was conducted in triplicate.

3.2.6 Ergocalciferol quantification

The concentration of ergocalciferol in the O/W nanoemulsions was measured using High-

performance liquid chromatography (HPLC). Ergocalciferol was extracted from the O/W emulsions prior to HPLC analysis using method previously described by Khalid *et al.*, (2015). 0.2 mL of nanoemulsion was mixed with 3.8 mL ethanol and vortexed for 2 min, followed by ultra-sonicating for 20 min. The samples were placed at room temperature for 30 min and filtered by passing through nylon membrane with a pore size of 0.45 µm before analysis. For the samples with the measurement below the limit of detection, 2 mL of filtered ethanolic extract was transferred into amber vial and dried by asperging nitrogen. After concentrating the samples and re-dissolving in 0.2 mL of ethanol by vortexing and mixing for 2 min, they were filtered using nylon filter. The concentration of ergocalciferol in the emulsions was evaluated by using the HPLC system (JASCO International Co., Tokyo, Japan) equipped with a UV-970 UV–Vis spectrophotometric detector, a PU-980 pump system, and an AS-2055 autosampler. C-18 reversed phase column (4.6×250 mm; Shimpack VP-ODS, Japan) was used as stationary phase and the column temperature was set at 35 ^{o}C. The mobile phase was composed of acetonitrile and methanol with the isocratic mixture ratio at 75:25 (v/v) and the flow rate was set at 1 mL min $^{-1}$. The injection volume was 20 µL and the detection wavelength was set at 265 nm. The concentration of ergocalciferol in samples was calculated by the standard curve and all the measurements were repeated in duplicate.

3.2.7 Statistical analysis

The statistical analysis was conducted according to the presented method in Section 2.2.4 (Chapter 2).

3.3 Results and discussion

3.3.1 Effect of pH on the stability of nanoemulsions

Food products and beverage typically exhibit broad range of pH, hence it is important to understand the influence of pH on the stability of ergocalciferol loaded nanoemulsions for future commercial applications. Fig 3.1a shows the change in $d_{4,3}$ of ML, SC and MO7S-stablized nanoemulsions as a function of pH. The obtained results showed $d_{4,3}$ of nanoemulsions stabilized by ML and MO7S remained constant over a wide range of pH. On the other hand, the $d_{4,3}$ of emulsions stabilized by SC increased sharply at pH 4 and 5 and these samples became highly unstable with an observed serum layer at the bottom and a creamed layer on the top of the tested tube (date not shown). We also examined the effect of pH on electrical characteristics (ζ-potential) of oil droplets (Fig. 3.1b). As reported previously, emulsions with ζ-potential greater than 30 mV (absolute value) were considered to be stable to oil droplet aggregation (Jacobs *et al.*, 2000). For SC-stabilized emulsions, the absolute values of ζ-potential were less than 30 mV at pH 4 and 5. These pH values were closed to the isoelectric point (pI) of SC, which was reported between pH 4.1 and 4.6 (Ching *et al.*, 2015, Zhang *et al.*, 2015c). Thus, the electrosteric repulsion of SC-coated droplets is not strong enough to keep the emulsion stable, at pH near to pI, since the attractive force between particles dominates electrosteric repulsion, thereby causing droplet aggregation. The high stability of SC-stabilized emulsions at other pH values were related to highly positive or negative charge of droplet at pH below or above the pI, respectively. Theoretically, MO7S as a non-ionic emulsifier was expected to have no charge. Nevertheless, we observed a negative charge on the MO7S-stabilized oil droplet at higher pH, and a considerable increase in ζ-potential as the pH was reduced to 2. The charge on the droplet was attributed to the presence of ionized impurities in MO7S, such as free fatty acids (Wang *et al.*, 2012b). Although, less charge (absolute value < 30 mV) was observed on the droplet and there was no increase in droplet size when MO7S

emulsions were adjusted to low pH. Unlike SC, the MO7S provides steric hindrance between droplets to stabilize the emulsions, and this steric repulsion is fairly insensitive to pH and ionic strength (McClements 2015). In the case of ML-stabilized emulsions, the high stability can be explained by the negative charge on the droplets across the entire pH range, which prevents emulsion droplets from aggregating.

3.3.2 Effect of ionic strength on the stability of nanoemulsions

It is imperative to see the stability of emulsified foods and beverages against ionic strength, as the emulsion-based products may be added with different amounts of mineral ions during product development. We therefore, investigated the influence of ionic strength on the physical stability of ergocalciferol loaded nanoemulsions. The addition of NaCl from 0 to 500 mM, resulted in no apparent changes in $d_{4,3}$ of nanoemulsions stabilized by MO7S and SC (Fig. 3.2a). MO7S stabilized nanoemulsions exhibited good stability in the presence of NaCl and was attributed to the non-ionic nature that forms steric repulsion between oil droplets and was fairly insensitive to the change in ionic strength as previously described in section 3.3.1 The addition of salt to SC-coated nanoemulsions resulted in destabilization of emulsions and was attributed to electrostatic screening effects (Israelachvili 2011). However, little increase in $d_{4,3}$ of SC-stabilized nanoemulsions was observed at highest concentration of NaCl (500 mM) in our study. This effect was probably due to the shielding effect of the salt on the electrostatic repulsion between SC-coated oil droplets was not strong enough (Fig. 3.2b). On the other hand, the casein molecules formed partial steric interaction between the droplets, which may be sufficiently large enough to prevent the droplet from growth. In contrast, a thin cream layer was occurred on the top of the ML-stabilized emulsions at ≥ 400 mM NaCl

(data not shown), and there was moderate increase in $d_{4,3}$ of the samples when NaCl concentration exceeded 300 mM. Similar, results have been reported by other researchers (McClements *et al.*, 2014). Previous studies indicated that the electrostatic screening effect of electrolytes might cause a significant reduction in electrostatic repulsion between droplets coated by ionic emulsifier at high salt concentration, which leads to the emulsion instability (Ozturk *et al.*, 2014, Tan *et al.*, 2016c). However, no apparent reduction in the magnitude of the ζ-potential was observed in ML-stabilized emulsions with increasing NaCl concentration. Therefore, the shielding effect due salt ions on the electrostatic repulsion between droplets could not explain the instability of ML-stabilized emulsions in our study. We predict that the electrolytes might change the hydrophilic−lipophilic balance and hydration conditions of the emulsifiers by depletion of the hydration shell around the polar head groups of emulsifier molecule (McClements *et al.*, 2014), which induces to the instability of ML-stabilized emulsions when exposed to high concentrations of NaCl.

3.3.3 Effect of freeze-thaw treatment on the stability of nanoemulsions

Many emulsion based food products like ice creams are exposed to fully or partially frozen state during their formulation, storage and utilization. The stability of these products during freeze-thawing is still a major challenge to food scientists and food industries (McClements 2015). Hence, in this section, we investigated the influence of freeze-thaw cycles on the stability of ergocalciferol-loaded nanoemulsions produced using different types of emulsifier was in the presence or absence of glucose.

Fig. 3.3a presented the formulation results without glucose in the continuous phase of O/W nanoemulsions. The results indicated that the ML-stabilized nanoemulsions showed

an appreciable increase in $d_{4,3}$ from 150 to 1000 nm after three cycles of freeze-thaw treatment. For the SC-coated nanoemulsions, the droplet size presented a slight increase in $d_{4,3}$ from 165 nm (initial) to 170 nm (3 cycles). However, the $d_{4,3}$ increased drastically to 14.47 μm even after one cycle of freeze-thawing for MO7S-stabilized nanoemulsions. Further freeze-thawing of MO7S-stabilized nanoemulsions showed extensive coalescence, leading to phase separation and oiling off (data not shown). These findings demonstrated that the stability of ergocalciferol loaded nanoemulsions towards freeze-thawing depends on the emulsifier type and follows the order of SC > ML > MO7S.

Crystallization of water takes place during freeze-thawing, as a result the emulsion droplets were forced to come closer to each other in the remaining non-frozen aqueous phase (Ghosh and Coupland 2008, Ogawa *et al.*, 2003). Thus, the emulsifier used should have the capability of protecting the emulsion droplet from coming closer and to stop the aggregation process. It is proven that the protein-stabilized emulsion had better freeze-thawing stability in comparison to those stabilized by the surfactants with smaller molecular weight. The proteins formed a thicker interfacial film around the droplets and inhibit the droplets from coalescence towards the freeze-thawing conditions (Cramp *et al.*, 2004, Palanuwech and Coupland 2003, McClements 2015). In our study protein based emulsifier (SC) protects the nanoemulsions better against freeze-thawing, in comparison to synthetic emulsifier (MO7S), which agrees well with the previous literatures. Tangsuphoom and Coupland (2009) found that SDS-stabilized coconut milk emulsion provides better freeze-thaw stability than those with Tween 20-coated samples. They reported that the association with SDS provided substantial electrostatic repulsion around lipid droplet and prevented the particles from coalescence, in comparison to Tween 20 during freeze-thawing.

In our study, ML was used as a small zwitter-ionic emulsifier to formulate the O/W nanoemulsions, similarly to SDS, it provides an electrostatic repulsion between lipid particles and prevents the droplet coalescence to some extent. Comparing to the nonionic surfactant (MO7S), this repulsion provided by ML is relatively stronger to slow down particle size growth when exposed to freeze-thaw treatment. In contrast, steric repulsion formed by MO7S is not strong enough to inhibit lipid droplet coalescence after freeze–thawing, ultimately resulting in creaming and oil phase separation as the freeze-thawing process progressed. On the other hand, no increase in $d_{4,3}$ or phase separation was observed in any of the samples, when glucose was added to the continuous phase (Fig. 3.3b). The preceding results indicated that nanoemulsions containing glucose had good freeze-thawing stability after 3 cycles, regardless of type of emulsifier used for stabilizing nanoemulsions. It is well known that the addition of sugars in O/W emulsions can effectively improve their freeze-thawing stability by reducing the amount of formed ice and prevent the droplets from coalescence (Ghosh *et al.*, 2006).

3.3.4 Effect of high temperature treatment on the stability of nanoemulsions

Thermal stability of emulsion-based delivery systems is important during processing and storage, since temperature always fluctuate specially during hot filling, pasteurization and autoclaving. We examined the effect of high temperature (80-120 ^{o}C) on the stability of ergocalciferol-loaded O/W nanoemulsions for a period of 1 h at specific temperature. For MO7S stabilized nanoemulsions (Fig. 3.4), the $d_{4,3}$ remains stable only at 80 ^{o}C, with further increase in temperature to 100 or 120 ^{o}C, a clear layer of oil phase was observed at the top of sample bottles (data not shown), which suggested that higher temperature induced the emulsions to droplet coalescence. As previously reported, dehydration of

surfactant hydrophilic head group occurred when heating the nonionic emulsifier-stabilized O/W emulsion close to or above the phase inversion temperature (PIT), which led to the change in the optimum curvature of the protective monolayer (Israelachvili 2011, McClements 2015). This progressive dehydration of emulsifier at the oil-water interface would lead to an ultralow interfacial tension, therefore facilitate the droplet coalescence and phase separation (Kabalnov and Wennerström 1996, McClements 2015). Presumably, this destabilization of MO7S-coated oil droplet due to the higher temperature (100 and 120 °C) used in this study is far above the PIT, which might have caused phase separation in emulsion system.

SC has been reported as a good protein emulsifier against high temperature due to its disordered structure. The emulsions stabilized by SC remained stable during 30 min of heating at 90 °C or 15 min of heating at 121 °C (Hunt and DALGLEISH 1995, Srinivasan *et al.*, 2002). As indicated in Fig. 3.4, an appreciably increase in mean droplet diameter was observed in our study only when SC-stabilized emulsion was heated at 120 °C for 1 h, which suggested that particle aggregation occurred due to heating. Our results somewhat agreed with a previous study reported by Chu *et al.*, (2008), in which 1 wt% SC-stabilized β-carotene nanodispersions showed an appreciably increase in particle size after heating them at 60 °C for 1h, and a rapid increase in size after heating for 4 h. The difference in results might be due to the longer heating time of 1 h in our study, rather than 15 min in the previous studies. The release of phosphorus from caseinate would take place when placing them at high temperature and dephosphorization lead to the reduction of negative charge on the caseinate and further promotes caseinate-caseinate interactions that is dependent on heating time and temperature (Chu *et al.*, 2008, Guo *et al.*, 1989, Howat and Wright 1934). The oil droplets growth was not observed in ML-stabilized

emulsions during the entire temperature range, which suggested that the electrostatic repulsion between ML-coated droplets was stronger enough to overcome the attractive interactions and prevent droplets aggregation at elevated temperatures.

3.3.5 Long-term storage stability on the stability of nanoemulsions

Long-term storage stability of ergocalciferol loaded nanoemulsions was investigated with different emulsifiers (ML, SC or MO7S) during storage at two different temperatures (25 or 55 ^{o}C) for a period of 30 days. This experiment was performed at neutral pH without salt addition.

3.3.5.1 Physical stability of nanoemulsions

Fig. 3.5 depicts the change in $d_{4,3}$ of ergocalciferol loaded nanoemulsions during 30 days of storage at 25 ^{o}C and 55 ^{o}C, respectively. All nanoemulsions stored at 25 ^{o}C exhibited good physical stability against oil droplet growth and phase separation and no change in their visible appearance and $d_{4,3}$ was observed during 30 days of storage (Fig. 3.5a). It is well accepted that nanoemulsions containing small droplet size are fairly stable due to the fact that Brownian motion of the nano-sized droplets can resist gravitational separation, flocculation and coalescence (Tadros *et al.*, 2004). On the other hand, soybean oil was used as dispersed phase in present study, due to long chain triglyceride structure, soybean oil is quite water-insoluble, hence limiting the effect of the Ostwald ripening (McClements and Rao 2011). The three different emulsifiers used in our study provided strong coated layer around the oil droplets and refrained from their growth during storage period of 30 days.

An accelerated test was performed to investigate the long-term physical stability of

nanoemulsions loaded with ergocalciferol by placing them at an elevated temperature (55 °C) for 30 days (Fig. 3.5b). In general, there is no distinct change in $d_{4,3}$ of nanoemulsions formed with ML and MO7S, suggesting that the emulsions stabilized by ML and MO7S were physically stable during 30 days of storage at 55 °C. In contrast, SC-stabilized nanoemulsions showed a slight increase in mean droplet size at the end of storage time, indicating that these nanoemulsions were slightly destabilized due to the long-term storage at an elevated temperature. The nanoemulsions prepared by SC were unstable to long-term storage at 55 °C, presumably attributed to caseinate-caseinate interactions, which were discussed in section 3.3.4.

3.3.5.2 Chemical stability of ergocalciferol

The long-term chemical stability of nanoemulsions stored at room temperature (25 °C) and at elevated temperature (55 °C) was evaluated by determining the change in retention of ergocalciferol during 30 days of storage. The fresh O/W nanoemulsions stabilized by ML, SC and MO7S contained 89.5 ± 3.1, 88.3 ± 3.2 and 91.7 ± 2.6 mg/L ergocalciferol, respectively. The resulting retention of ergocalciferol was regarded as 100% at day 0. In terms of ergocalciferol retention (Fig. 3.6), all the samples showed high chemical stability after storage at 25 °C for 30 days, with a slightly loss of 4.3%, 1.4% and 4.0% in ML, SC and MO7S-stabilized nanoemulsions, respectively. Oxidation of vitamin D in emulsion-based products would occur easier when they are exposed to light, in which light acts as catalyst (Banville *et al.*, 2000), as well as vitamin D is highly sensitive to high temperature. Thus, the high chemical stability of ergocalciferol in our study can be ascribed to the dark storage with mild storage temperature (25 °C).

Although, all nanoemulsions stored at 25 °C showed high chemical stability with

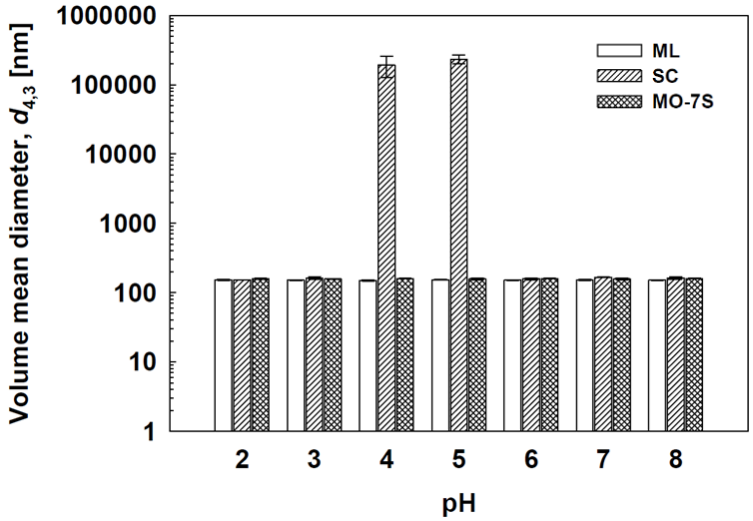
almost no loss of ergocalciferol during storage period, the effect of emulsifier on the degradation of ergocalciferol at elevated temperature (55 °C) was rather significant. Fig. 3.6b illustrates the influence of emulsifier type on ergocalciferol stability in nanoemulsions stored at 55 °C. After 30 days of storage, 54.4%, 4.5% and 49.0% of ergocalciferol retained in the nanoemulsions stabilized by ML, SC and MO7S, respectively. The data obtained from our study suggested that emulsifier type plays a vital role in maintaining the retention of ergocalciferol in nanoemulsions, and the loss of ergocalciferol decreased in the following order: SC >>MO7S> ML. In the literature, it was reported that protein emulsifiers, such as whey protein isolate (WPI) and SC, provide better chemical stability for encapsulated compounds, compared to emulsifiers with low molecular weight, due to the thick protective layers and anti-oxidative properties (Tan *et al.,* 2016c). Also it was reported that WPI exhibited better β-carotene chemical stability in nanoemulsions stored at 55 °C, comparing to those stabilized by small molecule surfactants such as Tween 20 and decaglycerol monolaurate (Mao *et al.,* 2009b). The obtained results in our study disagreed with those reported by Mao *et al.,* (2009a). The reasons for these opposite results are still unclear. However, it was thought to be related to the difference in protein emulsifier tested. The adsorbed SC molecules on the surface of oil droplet are less close to each other than WPI molecule, due to the higher flexibility of casein than globular whey protein isolates (M. Boland 2014). Thus, the most severe loss of vitamin D in SC-stabilized nanoemulsions, comparing to those coated with compacted emulsifier layer (small emulsifier or WPI) presumably attributed to huger gap between SC molecules induced ergocalciferol to be exposed to oxidation and degradation more quickly at an elevated temperature. Another reason that could explain SC-stabilized nanoemulsions exhibit lowest chemical stability in our study is that SC might undergo

thermal degradation (M. Boland 2014), resulting in weakening the membrane layer on the surface of oil droplet. Ergocalciferol in ML-stabilized nanoemulsions was least sensitive to thermal degradation among the samples and was attributed to phospholipids that play a role as anti-oxidizing agent, and play a vital role in encapsulation by reducing permeation of free radicals across the emulsion interface (Pan *et al.*, 2013).

3.4 Conclusions

The current study showed that ergocalciferol loaded nanoemulsions was successfully formulated via the high pressure homogenization using three different emulsifiers (ML, SC or MO7S). In addition, some of the major factors affecting the stability of resulting nanoemulsions were systematically evaluated. The stability of nanoemulsions to different environmental stresses were highly dependent on the emulsifier type, whereas no particular emulsifier tested was found to provide absolute stability to the nanoemulsions when exposed to pH, ionic strength, freeze-thaw cycles and high temperature treatment. Independent of emulsifier type, the nanoemulsions exhibited good physical and chemical stability during storage at 25 ^{o}C up to 30 days. In contrast, the long-term stability of nanoemulsions was highly dependent on the type of emulsifier tested at elevated temperature. The results of our study could provide useful information on the selection of appropriate emulsifier for producing stable nanoemulsions encapsulating functional bioactive compounds for commercial usage.

(a)



(b)

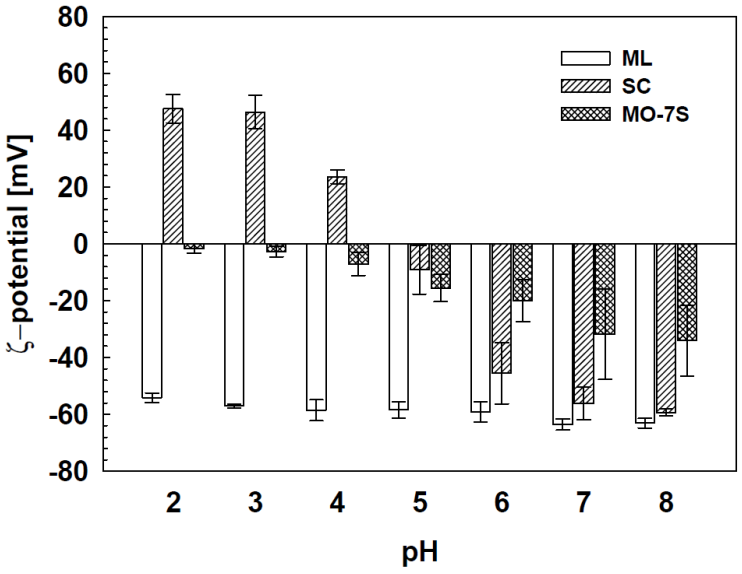
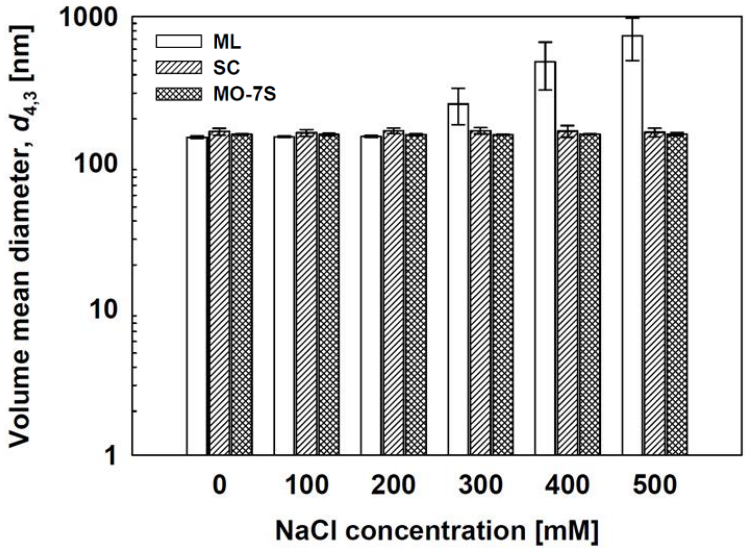


Fig. 3. 1: Effect of pH on the stability of O/W nanoemulsions. (a) $d_{4,3}$ and (b) ζ-potential of ergocalciferol loaded nanoemulsions stabilized by different emulsifiers.

(a)



(b)

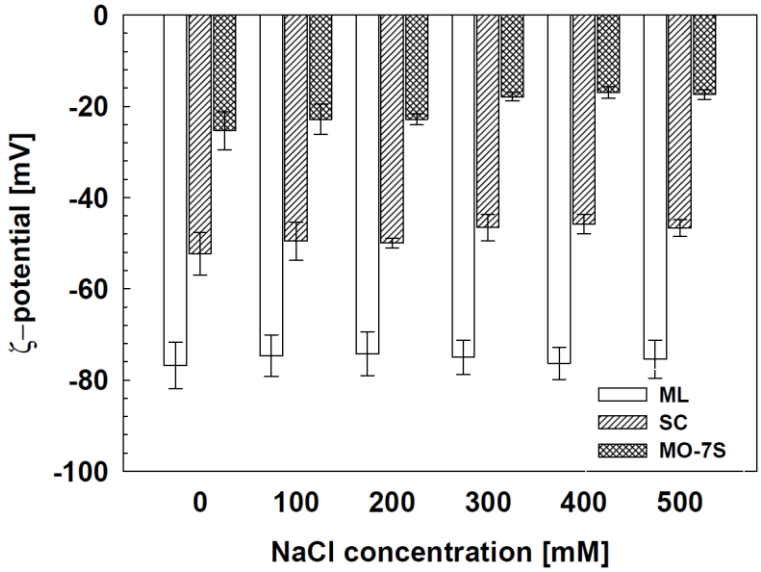
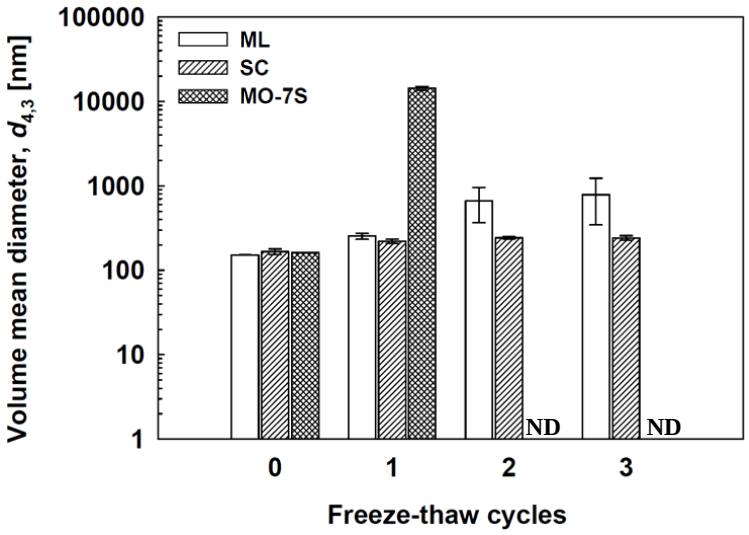


Fig. 3. 2: Effect of NaCl concentration on the stability of O/W nanoemulsions. (a) $d_{4,3}$ and (b) ζ-potential of ergocalciferol loaded nanoemulsions stabilized by different emulsifiers.

(a)



(b)

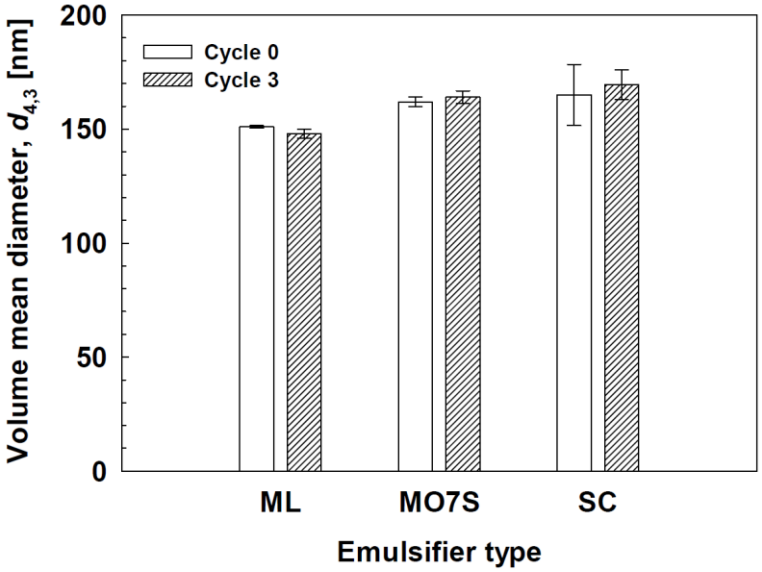


Fig. 3. 3: Effect of freeze-thawing on the stability of nanoemulsions loaded with ergocalciferol stabilized with different emulsifier types. (a) The change in $d_{4,3}$ of nanoemulsions without glucose, and (b) the change in $d_{4,3}$ of nanoemulsions with glucose. ND means not determination, because of oiling off.

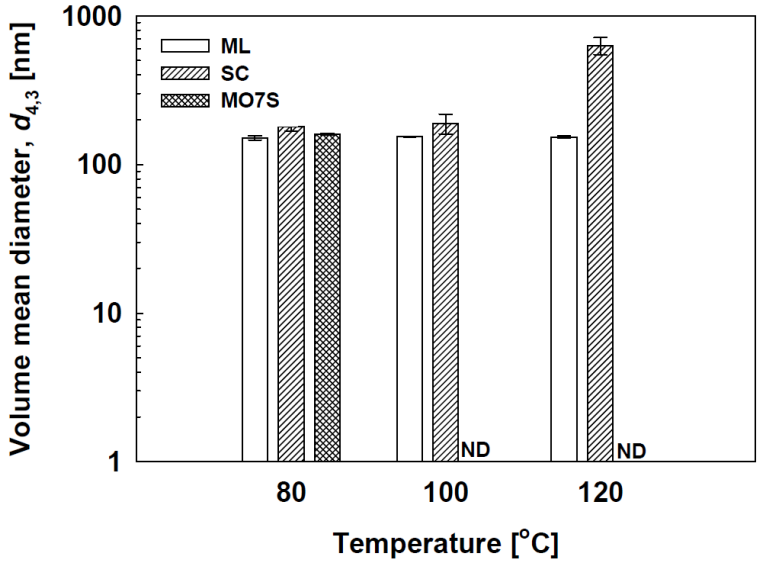
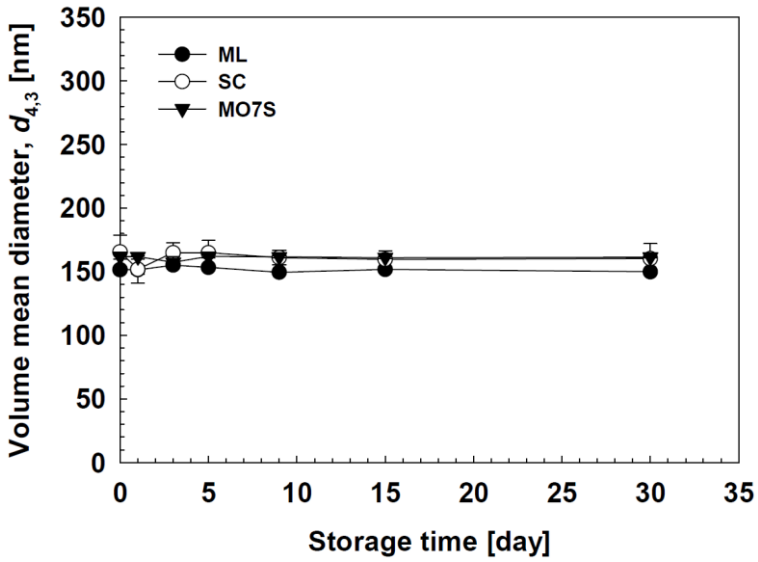


Fig. 3. 4: Effect of temperature on the stability of ergocalciferol-loaded nanoemulsions. ND means not determination, because of oiling off.

(a)



(b)

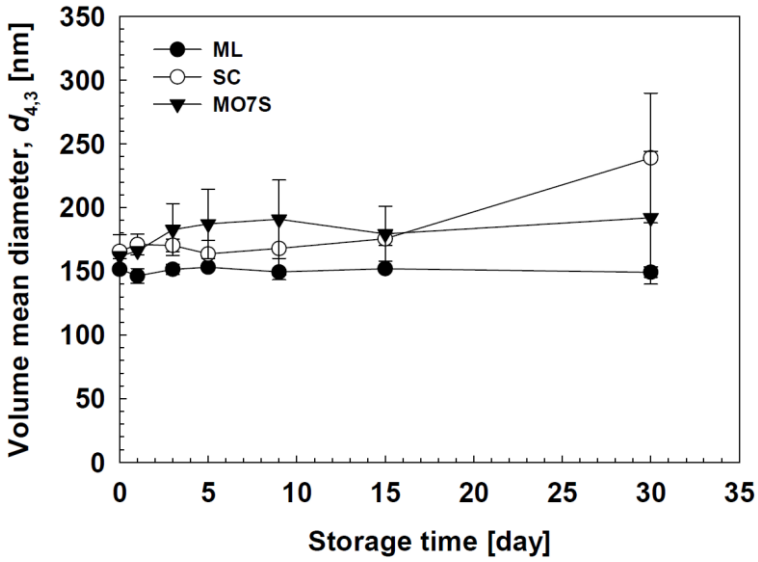
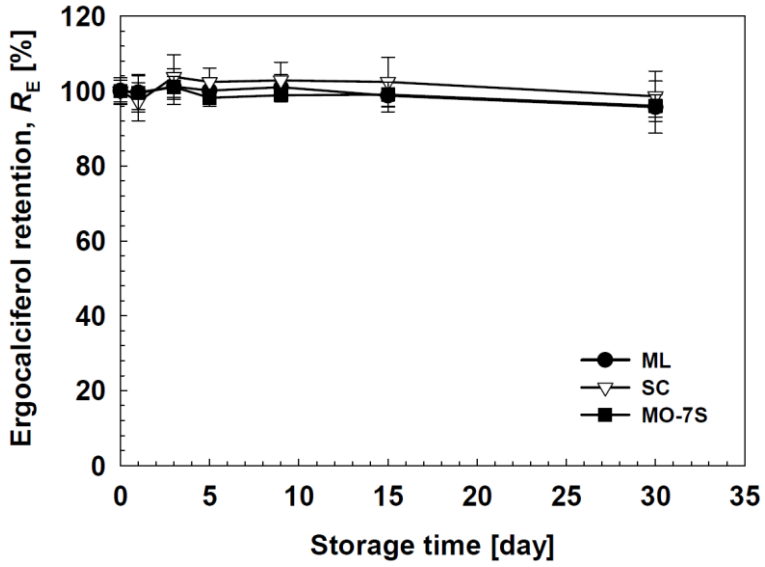


Fig. 3. 5: Effect of storage temperature on physical stability of ergocalciferol-loaded nanoemulsions stabilized by different emulsifiers: (a) 25 ^{o}C and (b) 55 ^{o}C.

(a)



(b)

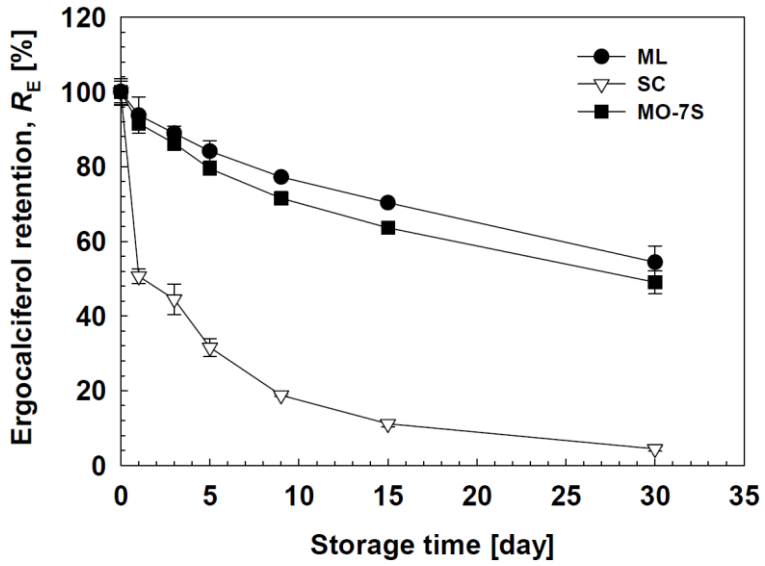


Fig. 3. 6: Effect of storage temperature on chemical stability of ergocalciferol-loaded nanoemulsions stabilized by different emulsifiers: (a) 25 °C and (b) 55 °C.

Chapter 4

In Vitro Digestion of Oil-in-Water Nanoemulsions Loaded with Ergocalciferol

4.1 Introduction

Vitamin D is widely known as a lipid-soluble compound that is essential to humans because this substance plays a crucial role in the absorption of calcium and maintenance of skeletal health (Holick 2004c, Khalid *et al.*, 2015). This bioactive compound may also provide a number of other health benefits, such as reducing the risk of certain cancers, inhibiting cardiovascular diseases, and improving immunity (Guttoff *et al.*, 2015). Vitamin D exists in two major chemical forms: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). In order to exert the functional effects, both these forms should be converted into their biologically active form, which is 25-dihydroxyvitamin D, via hydroxylation in the liver and kidneys (Christakos *et al.*, 2012). Cholecalciferol can be produced simply by exposing the human skin to sunlight, whereas ergocalciferol cannot be synthesized by the human body. In other words, ergocalciferol has to be supplied through food, such as mushroom, which is one of the major natural sources of ergocalciferol (Eitenmiller *et al.*, 2016). There are huge populations worldwide which are found to be vitamin D deficient due to a number of reasons: (i) insufficient sunlight exposure, (ii) extensive use of UV-protective cream, and (iii) poor intake of vitamin D-rich food (Guttoff *et al.*, 2015). In view of this problem, there has been an increasing interest in incorporating this lipid-soluble vitamin in food and beverage products. However, vitamin D incorporation poses certain problems related to its limited solubility in water and chemical instability against light, oxygen and high temperature (Luo *et al.*, 2012). Additionally, the relatively low absorption of vitamin D from food intake due to its poor water-solubility gives rise to another challenge in terms of designing functional foods with increased bioaccessibility.

Emulsion-based delivery system, especially nanoemulsions, is one of the more promising solutions to overcome these challenges, and has duly received a considerable amount of attention within the food industry. Oil-in-water (O/W) nanoemulsions are thermodynamically unstable systems that contain small oil droplets (d < 200 nm) dispersed in aqueous phase (Walker *et al.*, 2017). Nanoemulsions have better physical stability than conventional emulsions (d > 200 nm), which is due to Brownian movement can keep the oil droplets from creaming and eventually coalescing (Tadros *et al.*, 2004). In addition, small oil droplets are more effective at promoting the bioaccessibility of encapsulated lipophilic compounds than large emulsified droplets or non-emulsified oil (Liang *et al.*, 2013, Salvia-Trujillo *et al.*, 2013). Vitamin D-enriched nanoemulsions can be produced simply by dissolving this lipophilic substance in a carrier oil, and then homogenizing it with the aqueous phase containing a water-soluble emulsifier. As reported previously, there are various factors that influence the formation and stability of O/W emulsions loaded with vitamin D, including homogenization technique, carrier oil, emulsifier type and so on (Guttoff *et al.*, 2015, Khalid *et al.*, 2017, Khalid *et al.*, 2015). Besides affecting the formation and stability, some researchers have found that the initial carrier oil type and droplet size have significant impacts on the bioaccessibility of this functional ingredient delivered via nanoemulsion-based system (Ozturk *et al.*, 2015c, Salvia-Trujillo *et al.*, 2017). The bioaccessibility of encapsulated bioactive lipophilic component in a nanoemulsion is defined as the released fraction that is solubilized within the mixed micelles formed by phospholipids, bile salts and lipid digestion products (such as monoacylglycerols and free fatty acids) (Carbonell‐Capella *et al.*, 2014).

Emulsifier is one of the most crucial parameters to consider when producing a stable nanoemulsion. Emulsifier is able to adsorb onto the surface of droplets and act as a barrier

to protect the droplets from aggregation and coalescence during emulsification and storage (Shariffa *et al.,* 2016). In general, there are four different mechanisms that are responsible for the way an emulsifier stabilizes nanoemulsions: steric, electrostatic, electrosteric and electrostatic-steric mechanisms (Tan *et al.,* 2016c). Each category of emulsifier has its own advantages and disadvantages. In recent years, extensive studies on the effects of emulsifiers on the preparation, characteristics and stability of nanoemulsions have been widely reported. Since these aspects have been well-established, the next logical step would be the understanding of the biological fate of these nanoemulsions. However, to the best of our knowledge, there is still limited fundamental information on the bioaccessibility of vitamin D delivered via a nanoemulsion-based system, especially in the context of nanoemulsions fabricated using emulsifiers with different stabilizing mechanisms. Therefore, it would be interesting to investigate the influence of these emulsifiers on the release profile of encapsulated compounds, as well as to understand the changes in the gastrointestinal fate of the nanoemulsions during the digestion process.

Thus, in our study, ergocalciferol-loaded O/W nanoemulsions were prepared using either decaglycerol monooleate (MO7S), modified lecithin (ML), sodium caseinate (SC), or combined ML-MO7S, as emulsifier. MO7S (non-ionic) and ML (ionic) are small-molecule emulsifiers that possess steric and electrostatic stabilizing mechanisms, respectively. Meanwhile, SC is a surface-active protein with flexible structure that stabilizes the nanoemulsions by a combination of electrostatic and steric repulsion (electrosteric stabilizing mechanism) (Liu *et al.,* 2016, Tan *et al.,* 2016c). Finally, the combination of ML andMO7S (electrostatic-steric) was used to mimic the stabilizing mechanism of SC. Emulsifiers are critical components of a nanoemulsion and we believe

that the emulsifiers, each with its own stabilizing mechanism, will play an important role in the stability of emulsified droplet and protection and release of lipid-soluble bioactive compounds within the gastrointestinal tract. Therefore, the aim of this study was to study the impact of emulsifiers with different stabilizing mechanisms on the *in vitro* digestion and bioaccessibility of nanoemulsions loaded with ergocalciferol.

4.2 Materials and methods

4.2.1 Materials

Sodium caseinate (SC), refined soybean oil, HPLC grade ethanol, and α-amylase (015-26372) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Decaglycerol monooleate (MO7S) was provided by Sakamoto Yakuhin Kogyo Co., Ltd. (Osaka, Japan). Modified lecithin (ML; SLP WhiteLyso) was purchased from Tsuji Oil Mills Co., Ltd. (Tokyo, Japan). The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): HPLC grade methanol and acetonitrile, mucin from porcine stomach, Type II (M2378), pepsin from porcine gastric mucosa (P7000), pancreatin from porcine pancreas (P7545) and bile extract porcine (B8631). All other chemicals used in the present work were of analytical grade. Milli-Q water having a resistivity of 18 MΩ cm was used to prepare all the solutions and nanoemulsions.

4.2.2 Formulation of ergocalciferol-loaded O/W nanoemulsions

Ergocalciferol nanoemulsions containing 95 wt% of aqueous phase (Milli-Q water with 1 wt% of emulsifier) and 5 wt% of oil phase (soybean oil with 0.2 wt% ergocalciferol) were formulated by a high-pressure homogenization method (100 MPa, 4 Passes), which was described in Section 2.2.2 (Chapter 2). The emulsifiers used were ML, MO7S, SC or

ML-MO7S (ML: MO7S at a 1:1 ratio). The freshly produced nanoemulsions were then placed in a refrigerator (5 ^{o}C) for further analyses.

4.2.3 *In vitro* digestion

Ergocalciferol-loaded nanoemulsions prepared using different emulsifiers were subjected to an *in vitro* digestion model that simulated the mouth, stomach and small intestinal phases. The *in vitro* digestion model applied in our current study was based on those reported previously, with minor modifications (Qian *et al.*, 2012, Xia *et al.*, 2017).

4.2.3.1 Mouth phase

A simulated saliva fluid (SSF) containing 2.1g L^{-1} $NaHCO_3$, 0.117 g L^{-1} NaCl, 0.14 g L^{-1} KCl, 2.0 g L^{-1} α-amylase and 1 g L^{-1} mucin was prepared as described previously (Kong & Singh, 2008). Initial nanoemulsion sample (20 g) was mixed with an equivalent weight of SSF (20 g), and the mixture was adjusted to pH 7, if needed. The mixture was then shaken continuously at 100 strokes min^{-1} for 10 min using a temperature-controlled water bath at 37 °C (Personal-11, Taitec, Saitama, Japan).

4.2.3.2 Stomach phase

The simulated gastric fluid (SGF) was prepared by dissolving NaCl (2 g) and HCl (7 mL, 35-37%) in 1 L of Milli-Q water and then adding 3.2 g of pepsin. Sample collected from mouth phase (20 g) was added into 20 g of SGF, and the pH of the mixture was adjusted to 2.5 using 2.5 mol L^{-1} NaOH solution. Simulations of digestion in stomach phase was performed by shaking the resulting mixture at 100 strokes min^{-1} for 2 h at 37 °C.

4.2.3.3 Small intestinal phase

Thirty gram of sample collected from the stomach phase was adjusted to pH 7 using NaOH solution (2.5 M). Then, an aliquot (4 mL) of bile extract solution (46.8 mg mL^{-1} in phosphate buffer, pH 7) and 1 mL of calcium solution containing 110 mg of $CaCl_2$ were then added into the 30 g of digesta under magnetic stirring (100 rpm min^{-1}) at 37 ºC. The pH of the resulting mixture was adjusted to 7, if needed. Next, 2.5 mL of freshly prepared pancreatin suspension (24 mg mL^{-1} in phosphate buffer, pH 7) was added to the mixture. From this point, the pH of the samples was controlled manually to maintain it at pH 7 by adding NaOH solution (0.5 M) to neutralize the free fatty acids (FFA) released from the lipid during digestion. The volume of the added NaOH against time was recorded during 2 h of digestion in the small intestinal phase, and was then used to calculate the FFA release using the equation (4.1) (Ozturk *et al.*, 2015c).

$$\%\mathrm{FFA(t)} = \frac{\mathrm{V_{NaOH}(t)} \times \mathrm{M_{NaOH}} \times \mathrm{m_{Lipid}}}{2 \times \mathrm{W_{Lipid}}} \times 100 \quad (4.1)$$

where V_{NaOH} (t) is the volume (L) of NaOH solution titrated at digestion time (min), M_{NaOH} is the molarity of NaOH used (M), m_{Lipid} is the molecular weight of soybean oil (g/mol), and W_{Lipid} is the total mass (g) of soybean oil initially present in the samples during digestion in the small intestinal phase.

4.2.4 Droplet size and size distribution measurement

The measurement of droplet size was conducted according to the presented method in Section 2.2.3 (Chapter 2)

4.2.5 ζ-potential measurement

Prior to ζ-potential analysis, the samples from stomach phase were diluted 100 times with

water (pH 2.5), while all the other samples were diluted 100 times with phosphate buffer (5 mM, pH 7). And then the measurements were then conducted according to the presented method in Section 3.2.5 (Chapter 3).

4.2.6 Ergocalciferol content measurement

The content of ergocalciferol in the nanoemulsions was measured according to the presented method in Section 3.2.6 (Chapter 3).

4.2.7 Bioaccessibility and chemical stability evaluation

Ergocalciferol bioaccessibility in the nanoemulsion-based delivery system was determined by adopting a method described previously (Qian *et al.,* 2012), with some modifications. In brief, an aliquot of the raw digesta (10 mL) was collected after the samples passed through the small intestinal phase, and then centrifuged at 9,000 g for 60 min using a MX-307 centrifuge (Tomy Digital Biology Co., Ltd., Tokyo, Japan). Afterwards, an aliquot of the micelles phase was collected by passing the supernatant fraction through a syringe filter (0.45 μm). An aliquot of 0.5 mL raw digesta or micelles phases was mixed with 4.5 mL of ethanol, vortexed, ultra-sonicated for 30 min, and then filtered using a membrane filter (0.45 μm) before the ergocalciferol concentration analysis. The concentration of ergocalciferol in the raw digesta or micelles phases was then measured. The bioaccessibility and retention of ergocalciferol after digestion were calculated from the equations as follows:

$$\text{Bioaccessibility (\%)} = \frac{C_{Micelles}}{C_{Initial}} \times 100 \qquad (4.2)$$

$$\text{Retention (\%)} = \frac{C_{Digesta}}{C_{Initial}} \times 100 \qquad (4.3)$$

where $C_{Micelles}$ and $C_{Digesta}$ are the ergocalciferol contents recovered from micelles phase and raw digesta phase, respectively. $C_{Initial}$ is the ergocalciferol amount calculated in the initial nanoemulsion sample.

4.2.8 Microstructural analysis

Confocal scanning laser microscopy (Leica TCS SP8, Leica Microsystems Inc., Germany) was used to monitor the microstructural changes of samples before and after passing through each phase of digestion. Prior to confocal microscopy observation, 1 mL of each sample was mixed with 0.05 mL of Nile Red solution (1 mg mL^{-1} in ethanol) to stain the lipid phase. An oil-immersion objective lens was used to capture all microstructure images.

4.2.9 Statistical analysis

The statistical analysis was conducted according to the presented method in Section 2.2.4 (Chapter 2).

4.3 Results and discussion

4.3.1 Effect of emulsifier type on droplet characteristics

Initially, we investigated the influence of emulsifiers with different stabilizing mechanisms on the gastrointestinal fate of ergocalciferol-loaded nanoemulsions during digestion. Nanoemulsions were prepared using either MO7S, ML, SC or ML-MO7S as emulsifier. After each digestion phase, an aliquot of sample was taken for measurements of the droplet characteristics, including droplet size, size distribution, microstructure, and ζ-potential.

4.3.1.1 Initial nanoemulsions

The droplet size, $d_{4,3}$, was fairly small and similar for all the initial nanoemulsions stabilized using MO7S, ML, SC, or ML-MO7S: $d_{4,3}$ = 148, 149, 142, and 150 nm, respectively (Fig. 4.1). In addition, all the samples had monomodal size distributions without relatively large droplets in the initial system (Fig. 4.2). Confocal images showed that the oil droplets in these nanoemulsions were uniformly distributed, without evidence of droplet aggregation (Fig. 4.3). These results suggested that the homogenization conditions used in our study were capable of producing stable emulsions containing nano-sized droplets ($d_{4,3}$ < 200 nm), regardless of the emulsifier type. For the small-molecule emulsifiers (ML, MO7S and ML-MO7S), their adsorption on the oil droplet surface was fast during the homogenization process, leading to the formation of nanoemulsions with small droplet sizes (Mao *et al.,* 2009a). Protein-based emulsifiers are generally expected to result in larger droplet sizes due to their relatively lower absorption kinetics when compared with small-molecule emulsifiers (Mao *et al.*, 2009a, Tan *et al.*, 2016c). However, larger droplet size of SC-stabilized nanoemulsions comparing those stabilized by the other three emulsifiers was not observed in our study. For the SC-stabilized nanoemulsions, the mixture of surface-active casein molecule can form a thick layer with a combination of electrostatic and steric repulsion to prevent the newly-formed emulsion droplets from coalescing during the high-pressure homogenization, which presumably was responsible for the production of droplets with small sizes. The ζ-potential measurements revealed that all the initial electric charge of the nanoemulsions were of negative charge, with the magnitude highly dependent on the emulsifier type (Fig. 4.4). Relatively high ζ-potential values were obtained for nanoemulsions stabilized using ML,

SC and MLMO7S: -78, -50, and -65 mV, respectively. ML is an ionic emulsifier, and this intrinsic property of ML contributed towards the negative charge shown by ML-stabilized nanoemulsions. As for the case of SC-stabilized nanoemulsions, the ζ-potential will either be negative or positive charge depending on the pH of the nanoemulsions: pH either above or below the isoelectric point of SC (pI = 4.1-4.6) (Liu *et al.*, 2016, Shu *et al.*, 2016). Thus, in this study, the negative charge displayed by the SC-stabilized oil droplets could be attributed to the fact that the pH of primary nanoemulsions (around pH 7, data not shown) was much higher than the pI of SC. We anticipatedMO7S-coated oil droplets to possess an electric charge which is close to zero because MO7S is a non-ionic emulsifier. Unexpectedly, a moderate negative charge (-21 mV) was displayed by the MO7S-stabilized nanoemulsions (Fig. 4.4). This may be due to the presence of impurities, such as FFA, in the oil phase or surfactant (Qian *et al.*, 2012).

4.3.1.2 Mouth phase

After passing through the mouth phase, there was non-significant ($p > 0.05$) increase in $d_{4,3}$ and little change in droplet size distributions of the nanoemulsions stabilized byMO7S, ML, and ML-MO7S (Figs. 4.1 and 4.2). These results were also confirmed by confocal microscopy, which showed that the oil droplets remained small and no droplet flocculation or coalescence occurred during the 10 min of incubation in the artificial saliva (Fig. 4.3). The observed high stability of these nanoemulsions was possibly related to the steric hindrance provided byMO7S and/or electrostatic repulsion generated by ML. In comparison, the droplet size of SC-stabilized nanoemulsions slightly increased from 142 to 188 nm when the sample moved from the initial to the mouth phase. In addition, the droplet size distribution of SC-stabilized nanoemulsions showed a small amount of

population with slightly larger droplet size after those had similar droplet dimensions to the initial sample (Fig. 4.2). Minor droplet coalescence was also detected by confocal images as shown in Fig. 4.3, suggesting that SC-stabilized nanoemulsions became slightly unstable towards droplet growth when exposed to the oral phase. Previous studies have reported that the depletion and / or bridging flocculation caused by the presence of mucin in the artificial saliva could contribute to the growth of oil droplets in protein-stabilized emulsions (Sarkar *et al.*, 2009, Vingerhoeds *et al.*, 2005, Zhang *et al.*, 2015b). Generally, for all nanoemulsions, the ζ-potential remained fairly close to those displayed by the initial samples, even after being exposed to the artificial saliva (Fig. 4.4). The results indicated that the conditions of the mouth phase used in our study (neutral pH, and the presence of salt, mucin and α-amylase) had little impact on the ζ-potential of the nanoemulsions.

4.3.1.3 Stomach phase

The droplet characteristics of the nanoemulsions after gastric digestion were highly dependent on the emulsifier type. The MO7S-stabilized nanoemulsions did not show changes in the droplet size (Fig. 4.1), size distribution (Fig. 4.2), and microstructure (Fig. 4.3), suggesting that this emulsion system was highly stable against droplet aggregation under stomach conditions. The reason behind this observation could be related to the fact that the steric repulsion conferred by MO7S (a non-ionic emulsifier) is insensitive to the changes in pH, ionic strength, and protease activity (Zou *et al.*, 2015). Conversely, there was an appreciable increase in droplet size for the nanoemulsions stabilized by ML and SC (Fig. 4.1). Measurements of size distributions and confocal images also showed that a fraction of large oil droplets was present in ML- and SC-stabilized nanoemulsions (Fig.

4.2 and 4.3). This indicated that these two nanoemulsions were highly unstable towards droplet growth after exposure to the stomach phase. A previous study has also found that emulsions stabilized by non-ionic emulsifiers were more stable towards flocculation and coalescence under gastric condition than those prepared using protein- and phospholipid-based emulsifiers (Chang and McClements 2016). The instability of nanoemulsions stabilized by ML and SC under gastric phase was correlated with a series of physicochemical phenomenon: (i) the stomach phase reduced the electrostatic repulsion between ML and SC-coated droplets (as will be discussed later), thereby inducing aggregation; (ii) hydrolysis of the adsorbed protein layer by pepsin could possibly have reduced the ability of the protein layer to protect the droplets from growth; and (iii) the biopolymers might have promoted the depletion or bridging flocculation of oil droplets in the protein-stabilized nanoemulsions under acidic condition (Zou *et al.,* 2015). Meanwhile, the nanoemulsions stabilized by the combined emulsifiers (MO7S and ML) were more resistant towards droplet growth as compared to the ML-stabilized ones, suggesting that the physical stability of nanoemulsions stabilized by ML could be improved by the addition of MO7S. This phenomenon was presumably attributed to the combined effects of steric hindrance and electrostatic interaction could help to protect the oil droplets from aggregating under the simulated conditions of the stomach phase. The magnitude of negative charge in all the nanoemulsions was significantly ($p < 0.05$) decreased after exposure to stomach phase (Fig. 4.4). The reduction in ζ-potential between ML and ML-MO7S coated droplets could be relate to two reasons: (i) the presence of salt might cause the electrostatic effect; and (ii) the anionic phosphate groups on ML have pKa values near 1.5, which would lose their negatively charge at low pH (Carvalho *et al.*, 2014, Ozturk *et al.*, 2014). As mention earlier, the SC-coated droplets

would be expected to be highly positively charged at highly acidic condition (pH 2.5), because of this pH value was well below the pI of SC. The anionic mucin molecules could absorb onto the cationic protein emulsified droplets in stomach phase, which has been reported in recent studies (Zou *et al.,* 2015, Chang and McClements 2016). This effect may lead to the SC-stabilized nanoemulsions have a ζ-potential value near to zero.

4.3.1.4 Small intestinal phase

After 2 h of incubation in the small intestinal phase, a drastic increase in droplet size was observed in all samples (Fig. 4.1). Examination of the droplet size distribution revealed that the raw digesta for all the samples contained droplets with a broad size range (Fig.4.2). These measured droplets were presumably made up of a complex mixture of calcium salts sediment, non-digested oil, micelles, vesicles, and/or nondigested protein aggregates (Zhang *et al.,* 2015b). The microscopy images also indicated the presence of visibly large particle aggregates in all the samples (Fig. 4.3). However, there was clearly a difference in the nature of droplets present in the raw digesta for the nanoemulsions prepared using different emulsifiers (Fig. 4.3). A numerous of irregularly-shaped clumps of small particles were observed in the nanoemulsions stabilized by small-molecule emulsifiers (MO7S, ML, and ML-MO7S). On the hand, there were many spherical droplets with large sizes present for the SC-stabilized nanoemulsions. Apparently, a full lipid hydrolysis led to the generation of a mixtures of vesicles and micelles present in the small emulsifier-based nanoemulsions. On the other hand, partial digestion of lipid induced the coalescence between undigested oil droplets that was responsible for the large spherical droplets detected in the digesta of SC-based nanoemulsions. All samples exhibited relatively high negative electric charges after incubation in the small intestinal phase, as

seen in Fig. 4.4. The increase in the magnitude of negative charge on all the nanoemulsions could be attributed to several factors. Firstly, emulsifier molecules would be more negatively charged as the pH increased to 7 in small intestinal phase (Ozturk *et al.*, 2015c). Secondly, the absorption of surface-active anionic substance (bile salt and phospholipids from bile extract) on the droplets would contribute to a relatively high negative charge at neutral pH. Thirdly, the presence of anionic free fatty acids generated from lipid digestion would also lead to a relatively high negative charge in the small intestinal phase. Similar trend in the ζ-potential when the nanoemulsions moved from stomach phase to small intestinal phase was also reported by other authors (Xia *et al.*, 2017, Zhang *et al.*, 2015b).

4.3.2 Effect of emulsifier type on lipid digestion

The influence of emulsifier type on lipid digestion was determined by measuring the percentage of FFA released from the nanoemulsions in the small intestinal phase. According to Fig. 4.5a, there was an appreciable difference between the rate and extent of lipid digestion, depending on the emulsifier type used. The behavior of lipid digestion for the nanoemulsions stabilized by small-molecule emulsifiers (MO7S, ML and ML-MO7S) showed relatively similar trends: most of the emulsified triacylglycerols were digested by lipase within 20 min, after which the digestion rate slowly reduced until a relatively constant plateau was reached. Looking at these nanoemulsions individually, we found that the initial rate of FFA release decreased in the following order: MO7S > ML-MO7S > ML. There are two possible reasons behind the lowest initial lipid digestion detected in ML-stabilized nanoemulsions. Firstly, for the ML-stabilized nanoemulsions, extensive droplet flocs formed under gastric conditions, thus reducing the exposed surface

area and making it more difficult for pancreatic lipase to digest them when they move to the small intestinal phase (Zhang *et al.,* 2015a, Zou *et al.,* 2015). Secondly, it is known that calcium ion can bind with phospholipids, which is the main composition of ML (Sjoblom 2005). This interaction, to a certain extent, reduced the total amount of free Ca^{2+} present in the small intestinal phase, thereby decreasing the FFA release (Li *et al.,* 2011, Zhang *et al.,* 2015b). In the case of nanoemulsions stabilized by SC, the initial rate and extent of FFA release was much lower than in those prepared using the small-molecule emulsifiers (Fig. 4.5a). This finding was in agreement with previous studies reported that SC-stabilized emulsions revealed slow rate of lipid digestion in the small intestinal phase (Li *et al.,* 2012, Zhang *et al.,* 2015b). We believe the severe aggregation of SC-coated lipid droplets within the stomach phase, and the reduction of free Ca^{2+} due to calcium bridging between the caseins were the main reasons to explain its lowest initial rate of lipid digestion. In addition, the SC forms thick protective layer around the lipid droplets, which might also have inhibited the access of pancreatic lipase to the triacylglycerols. As indicated in Fig. 4.5b, there was an appreciable amount of undigested oil remaining in the SC-stabilized nanoemulsions after full digestion. This finding was further confirmed through the images obtained from the confocal microscopy (Fig. 4.3). We suppose this was because of lipase having insufficient time to fully access the SC-coated lipids due to the severe flocculation of the lipid droplets in the small intestinal phase.

4.3.3 Effect of emulsifier type on bioaccessibility and retention of ergocalciferol after digestion

4.3.3.1 Ergocalciferol bioaccessibility

The influence of emulsifiers with different stabilizing mechanisms on the bioaccessibility

of ergocalciferol was evaluated by measuring the ergocalciferol concentration in the micelle fraction. According to Fig. 4.6a, there were no significant ($p > 0.05$) differences in the ergocalciferol bioaccessibility between nanoemulsions stabilized using different small-molecule emulsifiers. We believe that this finding was possibly due to MO7S, ML and ML-MO7S emulsifiers generating similar percentages of FFA release at the end of the digestion process in the small intestinal phase. Conversely, the bioaccessibility of ergocalciferol in SC-stabilized nanoemulsions was only 12%, which was much lower than those shown by the other three nanoemulsions. Several potential reasons could be used to account for this phenomenon; (i) the lower amount of FFA released from SC-stabilized nanoemulsions resulted in lesser formation of mixed micelles available to solubilize the ergocalciferol, (ii) the oil droplets in the SC-stabilized nanoemulsions were not fully digested, therefore more ergocalciferol was retained within this undigested oil portion, (iii) the interaction between ergocalciferol and β-casein, one of the major protein fractions of SC, might have promoted the aggregation and precipitation of mixed micelles during digestion (Forrest *et al.*, 2005, Mun *et al.*, 2015), and (iv) MO7S and ML might participate in the formation of mixed micelles, thereby increasing the capacity to solubilize ergocalciferol.

4.3.3.2 Ergocalciferol retention

Finally, the stability of ergocalciferol after full digestion was also examined since it is one of the major factors affecting its bioaccessibility. According to Fig. 4.6b, almost no loss of ergocalciferol was observed in any of the samples, which demonstrated that this encapsulated oil-soluble vitamin was very stable against degradation under the digestion conditions. Previous studies also showed that β-carotene (a type of carotenoids with poor

chemical stability) were chemical stable in nanoemulsion-based system during the digestion process, which was attributed to the short digestion time (Qian *et al.*, 2012, Yi *et al.*, 2014).

4.4 Conclusions

The present work has shown the importance of emulsifier type in the ergocalciferol-loaded nanoemulsions on their potential biological fate (e.g., emulsion droplets stability, digestibility, and ergoaclciferol bioaccessibility and stability) within an *in vitro* digestion model. The physical stability of the prepared nanoemulsions during different phases of digestion was evaluated by measuring their droplet size, size distribution, ζ-potential and microstructure. The obtained results indicated that the physical stability of the nanoemulsions after passing through the mouth, stomach, and small intestinal phases was strongly dependent on the type of emulsifier used. Emulsifier type considerably affected the initial rate of FFA release from nanoemulsions by pancreatic lipases, and the results demonstrated that the access of pancreatic lipase to emulsified oil droplets decreased in the following order: MO7S > ML-MO7S > ML > SC.The nanoemulsions stabilized by small molecular emulsifiers (MO7S, ML and ML-MO7S) had similar ergocalciferol bioaccessibility, which may be related to the similar amount of lipid digestion products present. However, SC-stabilized nanoemulsions gave much lower ergocalciferol bioaccessibility than the other three nanoemulsions, which could be attributed to the fact that there was more undigested oil and less mixed micelles that solubilize the released ergocalciferol. The ergocalciferol remained stable in all samples, proving that the nanoemulsion-based delivery system was suitable to protect this oil-soluble vitamin from degradation under the *in vitro* digestion conditions. We are positively confident that the

findings obtained from this study will be important and useful for future designs of nanoemulsion-based delivery system to encapsulate lipophilic functional compounds, such as vitamin D.

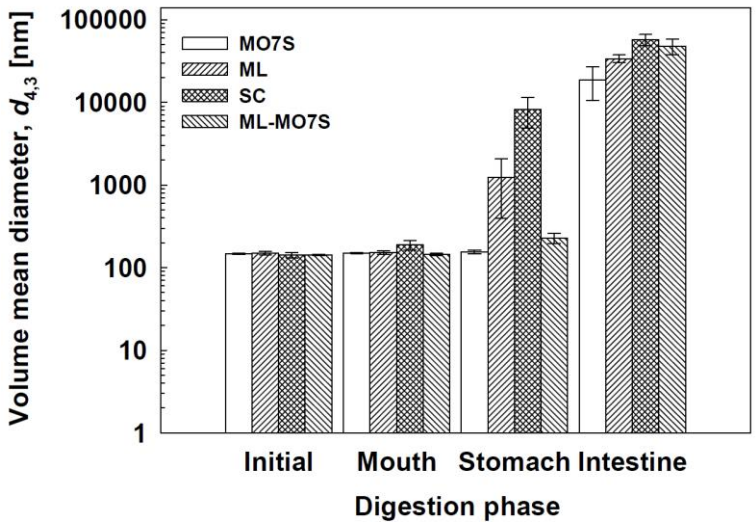


Fig. 4. 1: Effect of emulsifier type on volume mean diameter ($d_{4,3}$) of nanoemulsions loaded with ergocalciferol after exposure to different phases of the *in vitro* digestion model.

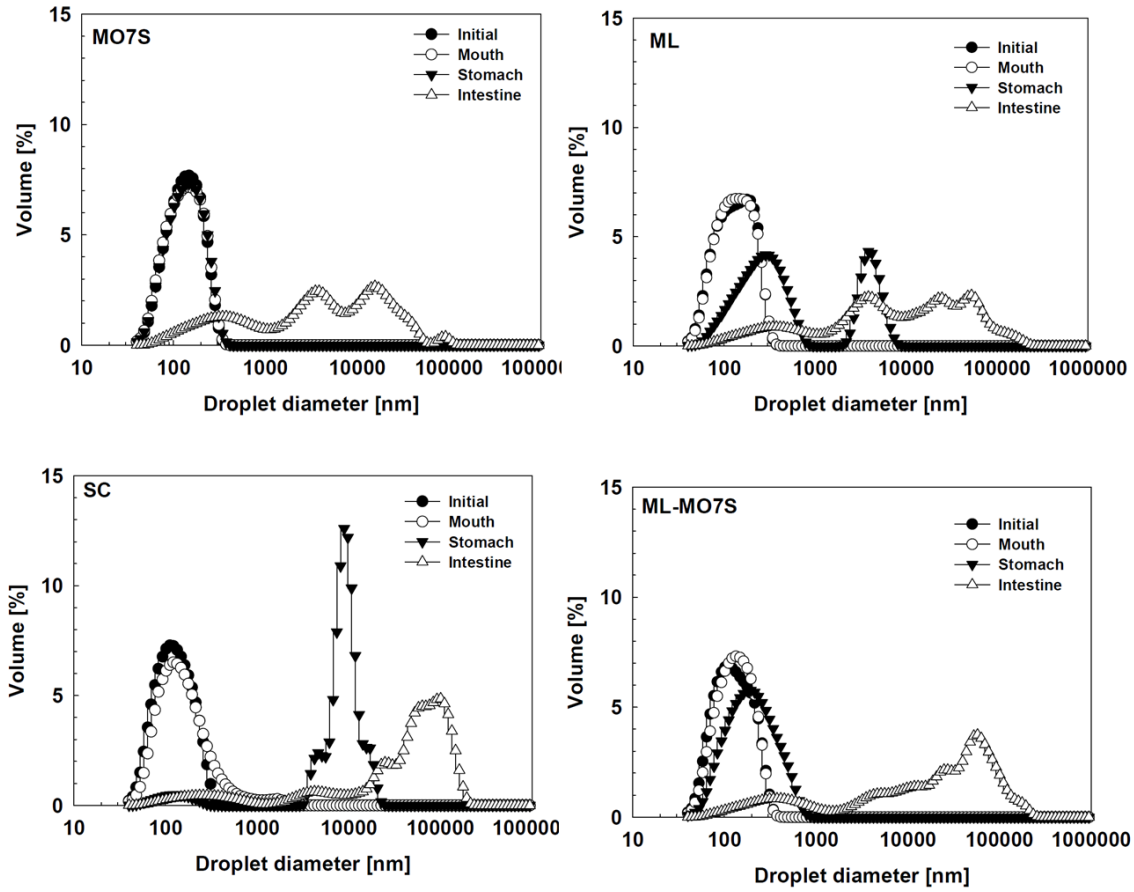


Fig. 4. 2: Effect of emulsifier type on droplet size distribution of nanoemulsions loaded with ergocalciferol after exposure to different phases of the *in vitro* digestion model.

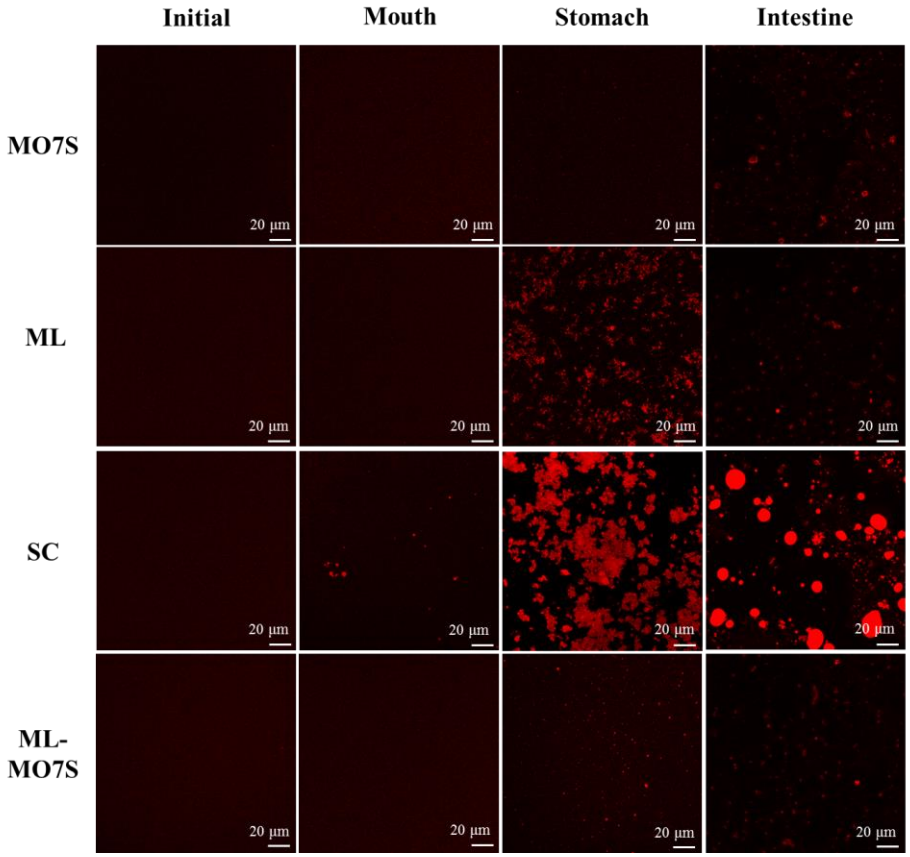


Fig. 4. 3: Effect of emulsifier type on the microstructure (confocal microscopy) of nanoemulsions loaded with ergocalciferol after exposure to different phases of the *in vitro* digestion model. (Scale bar = 20 µm).

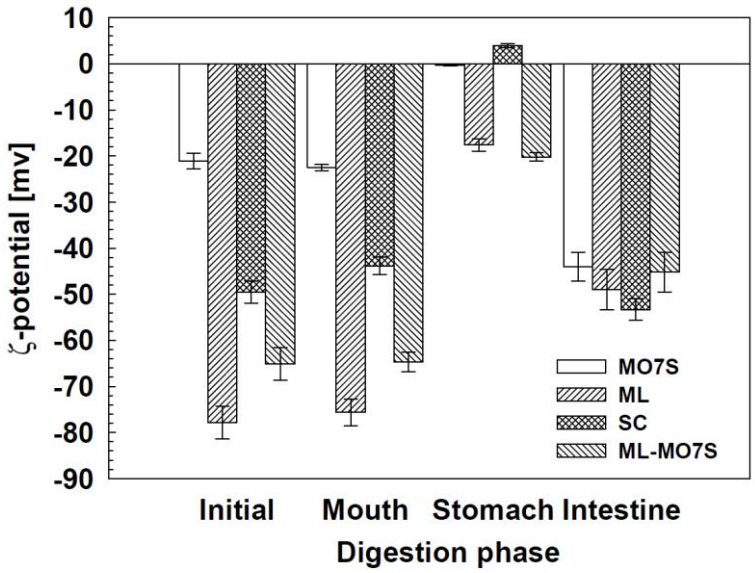
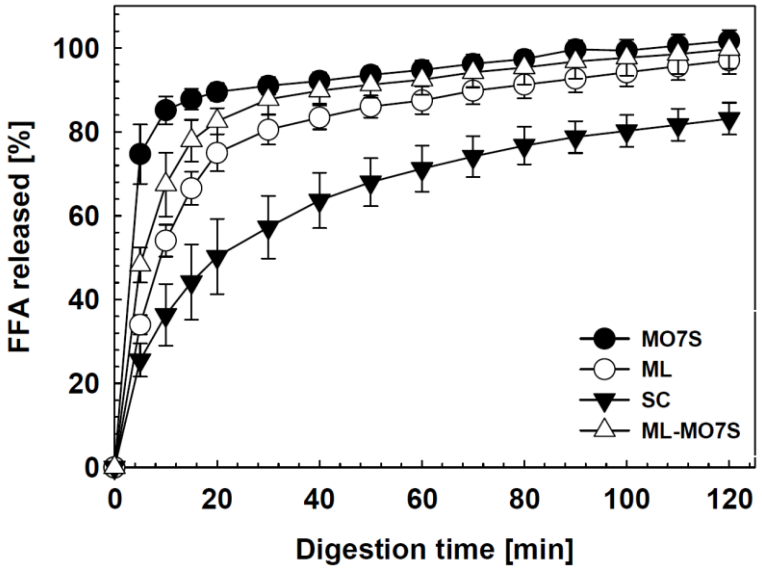


Fig. 4. 4: Effect of emulsifier type on the electric charge (ζ-potential) of nanoemulsions loaded with ergocalciferol after exposure to different phases of the *in vitro* digestion model.

(a)



(b)

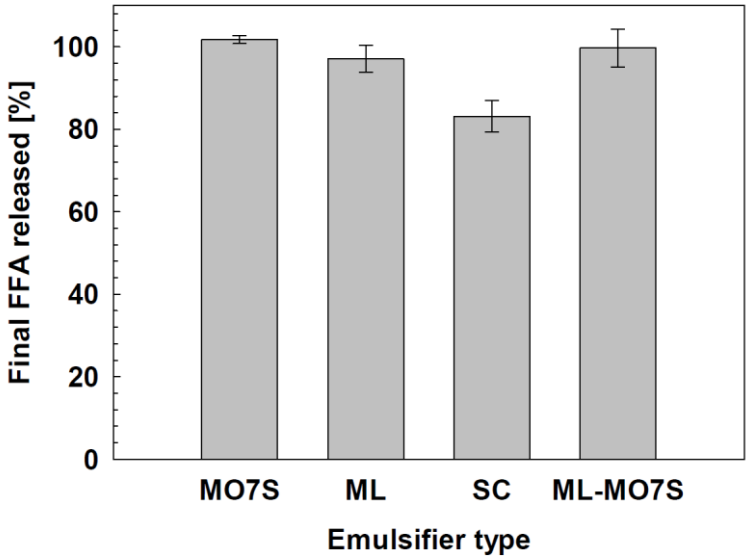
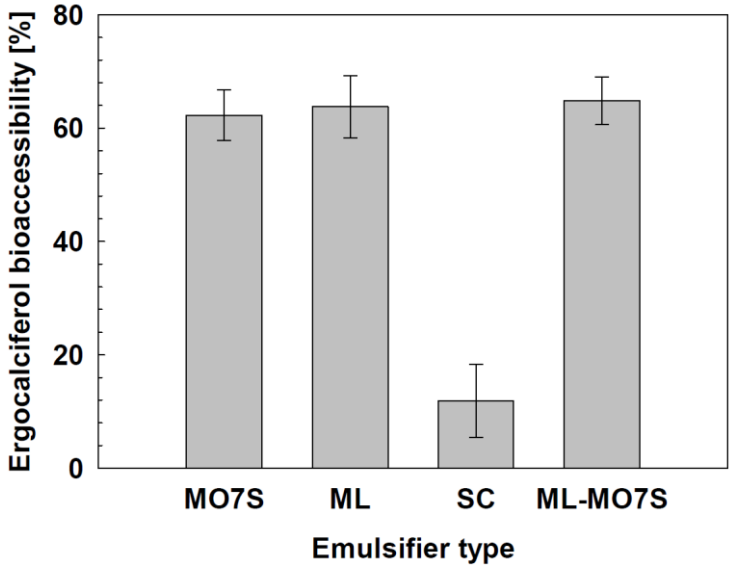


Fig. 4. 5: (a) Effect of emulsifier type on the release of free fatty acids (FFA) from nanoemulsions loaded with ergocalciferol during digestion in small intestinal phase. (b) Effect of emulsifier type on the final amount of FFA released after 2 h of small intestinal digestion.

(a)



(b)

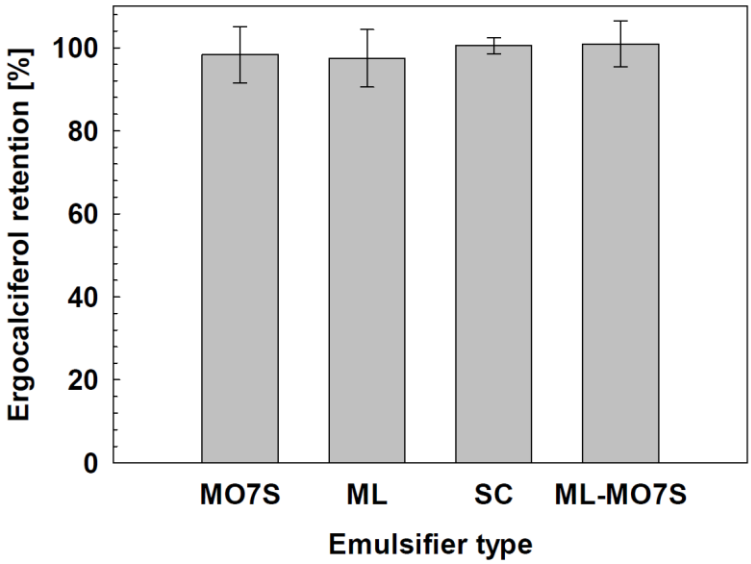


Fig. 4. 6: Effect of emulsifier type on the bioaccessibility of ergocalciferol in nanoemulsion-based delivery system. (b) Effect of emulsifier type on the chemical stability of ergocalciferol after full digestion.

Chapter 5

Formulation and Characterization of Ergocalciferol Nanodispersions Stabilized by Modified Lecithin and Sodium Caseinate

5.1 Introduction

Vitamin D is one of the essential bioactive compounds for human being, due to its special health-promoting functionality. Previous studies have demonstrated that this substance contributes to the development of bone, teeth and cartilage (Cranney *et al.*, 2008, Hark and Deen 2005). Moreover, it also prevents cancer, and enhances the heart and immune system (Haham *et al.*, 2012, Holick 2004c). Ergocalciferol is a type of plant-based vitamin D, which is naturally present in a low amount in wild mushrooms, whereas another type called cholecalciferol can be produced in the human skin via the exposure of sunlight (Guttoff *et al.*, 2015). However, there is still an estimated one billion people worldwide who either have vitamin D deficiency or insufficiency due to limited sun exposure, extensive UV-protecting sun cream usage, or poor dietary intake (Guttoff *et al.*, 2015, Khalid *et al.*, 2015). For these above-mentioned reasons, fortified food and beverage products with vitamin D are gaining attention in food industry nowadays. However, vitamin D has poor water-solubility, chemical instability towards environmental stresses and variable oral bioavailability (Haham *et al.*, 2012, Tsiaras and Weinstock 2011), which strongly limit the application of this vitamin as a functional ingredient to be incorporated into aqueous-based food products. In order to overcome these drawbacks in commercial usage, numerous efforts have been carried out to improve their water-solubility, stability and bioavailability through entrapment of this nutraceutical component into various types of colloidal delivery systems (Guttoff *et al.*, 2015, Abbasi *et al.*, 2014, Mohammadi *et al.*, 2014, Ozturk *et al.*, 2015c, Patel *et al.*, 2012).

Among these delivery systems, lipid-free nanodispersions consisting of fine nano-sized dispersed particles (20-200 nm) in aqueous phase have received great attention recently

in food, cosmetic and pharmaceutical applications, due to their high optical clarity and impressive improvement in solubility, stability and bioavailability (Anarjan *et al.*, 2015, Acosta 2009). There are two approaches, namely high-energy or low-energy methods, used to produce fine nanodispersions. In the high-energy approach, *e.g.* emulsification-evaporation method, certain expensive instrument such as high-pressure homogenizers, microfluidizers or ultrasonic probes are required to generate huge amount of disruptive forces to break large particles into nanoparticles. However, this approach might be undesirable for preparing nanodispersions containing heat-sensitive bioactive components due to the considerable generation of heat during the processing (Tan *et al.*, 2016b). Thus, the low-energy approach, including methods such as solvent displacement, emulsification-diffusion and spontaneous emulsification, is starting to gain popularity currently due to its simplicity and cost-effectiveness (Tan *et al.*, 2016b).

During the production of nanodispersions via either high-energy or low-energy methods, an emulsifier/surfactant is crucial for the formation of nanodispersed particles and to prevent them from aggregation against the destabilization process. In addition, it has been reported that this emulsifier layer could act as a barrier to protect the coated bioactive compounds from degradation by limiting the attack of oxidation inducers like free radicals and metal ions (Coupland and McClements 1996, Tan *et al.*, 2016c). Many previous studies used synthetic surfactants (such as polysorbates, polyglycerol ester of fatty acids, sodium dodecyl sulfate and so on) to produce nanodispersion-based delivery system for nutraceuticals with poor water-solubility (Tan *et al.*, 2016c, Tan and Nakajima 2005, Tan *et al.*, 2016a). However, consumers nowadays are demanding commercial food or beverage products containing 'label-friendly' ingredients in consideration of their health. Therefore, there is considerable interest in utilizing the more label-friendly

emulsifiers (such as natural emulsifiers) to produce nanodispersion- or nanoemulsion-based delivery systems. Sodium caseinate (SC), is a natural protein emulsifier, widely used as an effective emulsifying agent in the food industry. SC is a good alternative to synthetic surfactants, since it can facilitate the formulation of colloidal delivery systems and stabilize the emulsified droplets/particles against aggregation owing to a combination of electrostatic and steric repulsion (Liu *et al.,* 2016). In comparison to other protein emulsifiers (such as whey protein isolate and whey protein concentrate), SC has better thermal stability due to its relatively disordered structure (Chu *et al.,* 2008). Natural lecithin derived from soybean, egg or milk, should be modified via chemical or enzymatic techniques before it is effective at stabilizing emulsion/dispersion (Weete *et al.,* 1994). Modified lecithin (ML) is a zwitterionic emulsifier with effective emulsifying property, and provides high electrostatic repulsion to prevent the coated droplets/particles from growth. The ML emulsifier used in our work is an enzymatically modified phospholipid derived from the hydrolysis of soy lecithin. This kind of modified phospholipid could also be considered as a natural emulsifier, because both the materials and the enzymatic process used to produce the phospholipids is natural (van Hoogevest and Wendel 2014).

To the best of our knowledge, information related to the formulation of ergocalciferol nanodispersions using SC or ML via low-energy methods is still limited. In the current work, we aimed to produce ergocalciferol nanodispersions using the natural emulsifiers (SC or ML) via solvent displacement method, and then compare the stabilizing properties of the two emulsifiers against different environmental conditions (such as pH, ionic strength and thermal treatment) and during long-term storage at 4 ºC. In addition, the bioaccessibility of ergocalciferol nanodispersions in commercial lemon juice and milk as model system was also investigated using an *in vitro* gastrointestinal digestion model.

The study provides important information for utilizing natural emulsifiers in the development of label-friendly nanodispersion-based delivery system for water-insoluble vitamins or other hydrophobic functional compounds.

5.2 Materials and methods

5.2.1 Materials

Ergocalciferol, sodium caseinate, HPLC grade methanol, acetonitrile and ethanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Pepsin (from porcine gastric mucosa, P7000), pancreatin (from porcine pancreas, P7545) and bile extract (porcine, B8631) were purchased from Sigma Aldrich (St. Louis, MO, USA). Modified lecithin (SLP; WhiteLyso) was kindly provided by Tsuji Oil Mills Co. Ltd. (Tokyo, Japan). Lemon juice (commercial brand of C1000 Green Lemonade) and milk (commercial brand of Meiji Oishii Gyunyu) were purchased from a local supermarket in Japan. The lemon juice contains sodium (255 mg), carbohydrate (23g), vitamin C (1000 mg), citric acid (3000 mg) and polyphenol (10 mg) per 500 mL. The milk contains sodium (85 mg), calcium (227 mg), protein (6.8 g), fat (7.8 g), and carbohydrate (9.9 g) per 200 mL. Milli-Q water was used for the preparation of all the solutions and nanodispersions in the current study. All other chemicals used in this work were of analytical grade and used as such during experimentation.

5.2.2 Preparation of ergocalciferol nanodispersions

Ergocalciferol nanodispersions were produced by using solvent displacement method according to a previous study with certain modifications (Chu *et al.,* 2007). The Milli-Q water containing 0.1 wt% ML or SC were used as aqueous phase. Before preparation, SC

solution was centrifuged at 9,000 × *g* for 20 min at room temperature to remove any un-dissolved particles. The organic phase consisted of ergocalciferol at a concentration of 1 mg mL^{-1} in ethanol. The organic phase (5 mL) was then injected into 45 mL aqueous phase via a 22-gauge needle, under magnetic stirring at 550 rpm for 10 min. The flow rate of injection was set at 2.5 mL min^{-1} using a syringe pump (Model 11, Harvard Apparatus Inc., MA, USA). After preparation, the ethanol was immediately removed from the nanodispersions using a rotary evaporator (Eyela, Tokyo Rikakikai, Tokyo, Japan) under reduced pressure (67 hPa) at a temperature of 36 ºC for 45 min.

5.2.3 Nanodispersions stability testing

The stability of ergocalciferol nanodispersions when exposed to different environmental conditions and during long-term storage was investigated.

5.2.3.1 Effect of pH on the stability of ergocalciferol nanodispersions

Fresh nanodispersions were diluted with the same volume of phosphate buffer (5 mM, pH 7). The mixture was adjusted to different pH values (2-7) by using 1 mol L^{-1} HCl or 1 mol L^{-1} NaOH solution. The samples were then transferred to glass vials and stored at 4 ºC in the refrigerator for 24 h before analysis.

5.2.3.2 Effect of ionic strength on the stability of ergocalciferol nanodispersions

Fresh nanodispersions (2 mL) were placed in glass vials, and the final salt concentration adjusted to a range of 0-500 mM by adding 2 mL of salt solution (either NaCl or $CaCl_2$). Samples were later gently mixed and then stored at 4 ºC for 24 h prior to analysis.

5.2.3.3 Effect of thermal treatment on the stability of ergocalciferol nanodispersions

Fresh nanodispersions (5 mL) were transferred to a glass test tube and sealed well using a metallic cap. The samples were placed in an autoclaving device (KTS-2346, ALP Co., Ltd, Tokyo, Japan) at 120 ºC for 0-60 min. The mean particle size was then measured after cooling at room temperature.

5.2.3.4 Storage stability of ergocalciferol nanodispersions

The nanodispersions were added with sodium azide (0.02 wt%) as an antimicrobial agent to inhibit any microbial growth. Samples were then transferred to glass tubes and incubated at 4 ºC for 30 days under dark condition. The particle size and ergocalciferol content in the nanodispersions system were measured at 10-day intervals.

5.2.4 Ergocalciferol quantification

The content of ergocalciferol in the nanoemulsions was measured according to the presented method in Section 3.2.6 (Chapter 3).

5.2.4 *In vitro* gastrointestinal digestion

The ergocalciferol nanodispersions, after overnight storage at 4 ºC, were used for experiments in this section. The nanodispersions were diluted with the same volume of lemon juice, milk or phosphate buffer (5 mM, pH 7) before they were passed through a two-step digestion model. We applied this *in vitro* gastrointestinal digestion model, which simulates the gastric and small intestinal conditions, according to a previous study, with minor modifications (Mun *et al.*, 2015).

Stage I (stomach phase): The fresh simulated gastric fluid (SGF) was prepared by dissolving 2 g of NaCl and 7 mL concentrated HCl (35-37%) with Milli-Q water up to 1 L and adding 3.2 g of pepsin. The diluted ergocalciferol nanodispersions (15 mL) in lemon juice, milk or phosphate buffer as mentioned above were mixed with 15 mL of SGF and then the pH was adjusted to pH 2.5 by using 2.5 mol L^{-1} NaOH. The resulting samples were then maintained at 37 °C with continuous shaking at 100 strokes min^{-1} for 2 h in a Personal-11 water bath (Taitec, Saitama, Japan).

Stage II (small intestinal phase): The samples from the stomach stage were adjusted to pH 7 using 2.5 mol L^{-1} NaOH. Then, 3.5 mL of freshly prepared bile extract solution (187.5 mg/3.5 mL in phosphate buffer, pH 7) and 1.5 mL of salt solution (150 mM NaCl and 10 mM $CaCl_2$ in Milli-Q water) were added into the samples. The mixture was then adjusted back to pH 7, followed by the addition of 2.5 mL freshly prepared pancreatin suspension (187.5 mg/2.5 mL in phosphate buffer, pH 7). The samples were then incubated with continuous shaking at 100 strokes min^{-1} for 2 h in the water bath with temperature controlled at 37 °C. For the diluted nanodispersions in milk, NaOH (2.5 mol L^{-1}) was added at 30 min intervals to neutralize the free fatty acid released from the milk lipid digestion and maintain the condition at pH 7.

5.2.5 Particle size, size distribution and polydispersity index (PDI) measurement

The measurements of particle size, size distribution and PDI of resulting ergocalciferol nanodispersions were obtained via dynamic light scattering technique using Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). The nanodispersions without further dilutions were automatically measured at 25 ºC. The refractive indexes for ergocalciferol and water were set at 1.51 and 1.33, respectively. The intensity of the

scattered light was analyzed at an angle of 173º (non-invasive backscatter detection). The particle size of the nanodispersions was expressed as Z-average mean diameter (d_{av}). The measurement of each sample was repeated thrice.

5.2.6 ζ-potential measurement

The ζ-potential measurements of ergocalciferol nanodispersions without further dilution were conducted according to the presented method in Section 3.2.5 (Chapter 3).

5.2.7 Ergocalciferol bioaccessibility and stability after digestion

The Ergocalciferol bioaccessibility and stability after digestion were conducted according to the presented method in Section 4.2.7 (Chapter 4).

5.2.8 Statistical analysis

The statistical analysis was conducted according to the presented method in Section 2.2.4 (Chapter 2).

5.3 Results and discussion

5.3.1 Formulation of ergocalciferol nanodispersions

Fig. 5.1 shows the d_{av}, PDI and size distribution of ergocalciferol nanodispersions stabilized by ML and SC. The results indicated that both ML- and SC-stabilized nanodispersions have monomodal size distribution. The nanodispersions produced using ML had smaller d_{av} of 56 nm with a PDI of 0.213 in comparison to the SC-stabilized nanodispersions which showed a d_{av} of 112 nm and PDI of 0.137. During solvent displacement process, ML and SC molecules in the aqueous phase adsorbed onto the

surface of ergocalciferol particles, and then formed a protective layer to prevent the particles from aggregation. The observed differences in d_{av} between ML- and SC-stabilized nanodispersions were related to the difference in the composition of the two emulsifiers. SC is a natural biopolymer containing larger molecules that adsorb more slowly onto the surface of ergocalciferol particles than emulsifier with smaller molecules such as ML. The lower adsorption rate of emulsifier could induce relatively higher coalescence during solvent diffusing process, thus leading to bigger d_{av} in SC-stabilized nanodispersions. In addition, SC forms thicker interfacial layer around the particles, which also resulted in nanodispersions with bigger d_{av} than those formulated with ML (Ozturk *et al.,* 2015a).

5.3.2 Effect of pH on the stability of ergocalciferol nanodispersions

The effect of pH on the changes in d_{av} and ζ-potential of ergocalciferol nanodispersions was investigated. Fig. 5.2a indicates that nanodispersions prepared using SC were only stable against particle size aggregation at pH 2 to 3 and 6 to 7. However, the nanodispersions became opaque with the d_{av} increasing notably at pH 4. The nanodispersions were highly unstable to phase separation with a white precipitation observed at the bottom of the glass vials at pH 5. Fig. 5.2b shows the ζ-potential of SC-stabilized particles with function of pH that provided some insight into the nanodispersions instability. The SC-stabilized nanodispersions exhibited positive charge well below the isoelectric point of caseinates (pI≈4.6) and highly negative charge above the pI. For SC-coated nanodispersions, the stability was primarily maintained by the strong electrostatic repulsion (combined with minimal steric hindrance) between the particles. Thus, particle flocculation occurred in SC-stabilized nanodispersions when the

electrostatic repulsion is not strong enough to overcome the attractive interactions (e.g., van der Waals and hydrophobic) acting between the particles at pH close to the pI. On the contrary, pH had little impact on the stability of ML-stabilized ergocalciferol nanodispersions, since their particle size did not increase across the range of pH as indicated in Fig. 5.2a. The good stability of ML-coated nanodispersions against different pH was due to the strong electrostatic repulsion between the highly negative charged particles, which was confirmed in Fig. 5.2b. The high negative charge allowed the particles to constantly repel one another, leading to inhibition towards particles aggregation.

5.3.3 Effect of ionic strength on the stability of ergocalciferol nanodispersions

The addition of minerals into foods and beverages may occur in commercial applications. Therefore, we examined the stability of ML- and SC-stabilized nanodispersions in the presence of salt such as NaCl or $CaCl_2$ at different concentrations (0-500 mM).

5.3.3.1 Effect of NaCl on the stability of ergocalciferol nanodispersions

All the samples exhibited excellent stability against NaCl addition up to 500 mM, without showing any significant changes in visible appearance and d_{av} (data not shown). For the nanodispersions containing SC-stabilized particles, similar result was reported in a previous study whereby 0.1% SC stabilized lutein nanodispersions were stable against NaCl concentration (0-500 mM) (Tan *et al.*, 2016c). On the other hand, it has been reported that extensive particle size growth occurred at > 150 mM NaCl for the β-carotene nanodispersions stabilized using 1% SC (Chu *et al.*, 2008). The observed difference in these results might be related to the differences in SC concentration used in the two

studies. The SC concentration used in their study (Chu *et al.,* 2008) was much higher than the one used in our study. It is presumed that the large amount of free caseinate molecules in the aqueous phase might undergo consecutive self-association with increasing NaCl concentration, and exist as aggregates in the nanodispersions (Swaisgood 2003).

5.3.3.2 Effect of $CaCl_2$ on the stability of ergocalciferol nanodispersions

Fig. 5.3a depicts the effect of $CaCl_2$ addition on the changes in particle size of nanodispersions produced using ML and SC. In general, all the nanodispersions stabilized by ML became unstable with a slight increase in d_{av} when they were subjected to solution containing $CaCl_2$. The samples were most sensitive to particle growth at the lowest tested $CaCl_2$ concentration (100 mM), whereas the particle size slightly decreased when the salt concentration was increased from 100 to 500 mM. As mentioned before, the ML provides electrostatic repulsive forces between the particles to prevent them from coming closer together and thus, preventing aggregation. The addition of divalent Ca^{2+} ions could screen the negative charge, and therefore reduce electrostatic repulsion between the particles, which led to the instability of ML-stabilized nanodispersions. This explanation was confirmed by Fig. 5.3b, which shows a decrease in the magnitude of ζ-potential with increasing $CaCl_2$ concentration in ML-stabilized nanodispersions. On the other hand, the binding of Ca^{2+} ions to zwitterionic phospholipids at higher concentration could form thicker layer (Sjoblom 2005), which could explain the slight increase in the stability of ML-stabilized nanodispersions with further increasing salt concentration. In comparison, SC-stabilized nanodispersions became opaque and showed significant increase in particle size with increasing $CaCl_2$ concentration. The instability was attributed to the screening effect of Ca^{2+} ions on the electrostatic repulsion between SC-coated particles as described

in this section earlier, and this is further confirmed by the drastic drop in the ζ-potential of SC-stabilized nanodispersions (Fig. 5.3b). In addition, Ca^{2+} addition could induce calcium bridges between the protein emulsifier and promote extensive aggregation in the SC-stabilized nanodispersions (Teo *et al.,* 2016).

5.3.4 Effect of thermal treatment on the stability of ergocalciferol nanodispersions

The influence of thermal treatment on the physical stability of ergocalciferol nanodispersions was tested in this section. Fig. 5.4 illustrates that ML-stabilized nanodispersions were stable against high temperature, since the particle size remained constant across the entire range of heating time. Probably, the electrostatic repulsion between ML-stabilized nanoparticles was still strong enough to prevent them from coming closer together, even during 60 min of heating at 120 ºC. In comparison, SC-stabilized nanodispersions became unstable after thermal treatment, with an appreciable increase in particle size from 107 nm to 174 nm after 60 min of heating. In addition, a slight decrease in transparency was observed in SC-stabilized nanodispersions with increasing heating time, which was attributed to the increase in the particle size. The reason behind the thermal instability of SC-stabilized nanodispersions is that heating induced the dephosphorylation of serine phosphate groups in the caseinate molecules, which then led to a reduction in the negative molecule charge, thus promoting casein–casein interaction (Guo *et al.,* 1989).

5.3.5 Long-term storage stability of ergocalciferol nanodispersions

It is imperative to determine the shelf life of nanodipersions for potential food and beverage applications. Thus, we investigated the long-term physicochemical stability of

ergocalciferol nanodispersions formed using ML or SC during storage at 4 ºC for 30 days. These tests were performed using the nanodispersions at neutral pH without salt addition.

5.3.5.1 Physical stability of ergocalciferol nanodispersions during storage

Fig. 5.5a shows that there was no distinct increase in the d_{av} for ML-stabilized nanodispersions after 30 days of storage at 4 ºC. There are several factors that explain the high long-term physical stability of nanodispersions prepared using ML. Firstly, the Brownian motion of tiny particles in ML-stabilized nanodispersions have the capability of overcoming the gravitational separation force that usually leads to nanodispersions instability (Tadros *et al.*, 2004). Secondly, ergocalciferol as a water-insoluble compound, limits the destabilizing effect of Ostwald ripening (McClements and Rao 2011). Thirdly, the negative charge of ML-stabilized ergocalciferol particles was sufficient to inhibit particles from aggregating with each other during the tested period of storage. In comparison, we observed a slight increase in d_{av} for SC-stabilized nanodispersions during the storage period. Nevertheless, the overall increase in d_{av} after 30 days of storage was relatively small (from 122 nm to 134 nm), thus we consider them to be generally stable. The most likely reason behind the observed slight increase in the d_{av} of SC-stabilized nanodispersions was depletion flocculation caused by the presence of unadsorbed caseinate in the aqueous phase (Dickinson and Golding 1997). The slow growth rate was ascribed to the small particle size and the slow rate of Ostwald ripening in SC-stabilized nanodispersions as described earlier. In addition, the strong electrostatic surface potential combined with some steric repulsion between the particles formed using SC could also inhibit the particles from aggregation.

5.3.5.2 Chemical stability of ergocalciferol during storage

The initial ergocalciferol concentrations in ML- and SC-stabilized nanodispersions were 125 ± 4 and 119 ± 5 mg/L, respectively (data not shown). Fig. 5.5b shows the changes in ergocalciferol retention of nanodispersions produced using ML and SC during storage at 4 ºC. As expected, the nanodispersions containing SC-coated particles had good chemical stability, with only slight ergocalciferol loss of 8.5% at the end of the 30 days of storage. This result is in agreement with a previous study that evaluated vitamin D_3-loaded casein micelles prepared by using ultra-high-pressure homogenization. Only a slight loss of vitamin D_3 (≈10% loss) was observed over a period of 28 days at 4 ºC (Haham *et al.*, 2012). It is well established that protein emulsifier is effective at protecting encapsulated bioactive compounds with low stability (such as lutein and β-carotene) from degradation and oxidation due to its ability to form thick interfacial layer on the particles and iron-chelating property (Tan *et al.,* 2016c, Yin *et al.,* 2009). We expected ergocalciferol to be more susceptible to degradation in nanodispersions with smaller particle size. We reasoned that smaller particle size means a larger specific surface area, and this would cause ergocalciferol to be exposed to oxidation or decomposition more quickly. However, in our study, no sign of ergocalciferol loss was observed in ML-stabilized nanodispersions, although the particle size was much smaller than those in SC-stabilized nanodispersions. This could be due to the fact that phospholipids emulsifiers can also act as an effective anti-oxidizing agent to retard the loss of ergocalciferol by reducing permeation of free radicals across the particle interface (Pan *et al.*, 2013). Overall, the results suggested that both ML and SC are excellent natural emulsifiers that may be suitable for producing ergocalciferol nanodispersions with long-term storage stability.

5.3.6 Ergocalciferol bioaccessibility and stability after digestion

Nanodispersion-based system for vitamin D or other bioactive compounds could be incorporated into other water-based products such as fruit juice and milk. Therefore, we investigated the influence of emulsifier type (ML and SC) and the incorporation of lemon juice or milk on the bioaccessibility and stability of ergocalciferol nanodispersions using an *in vitro* gastrointestinal digestion model. The nanodispersions with the addition of phosphate buffer was used as control. In the absence of milk, ML-stabilized nanodispersions presented much higher bioaccessibility (≈70%) as compared to those stabilized by SC (≈40%), which suggested that the nature of interfacial layer coating on the particles has a major impact on their bioaccessibility (Fig. 5.6). A previous study also showed similar result whereby curcumin nanoparticles exhibited low bioaccessibility (<35%) when coated with zein (another type of protein) (Zou *et al.*, 2016). The difference in ergocalciferol bioaccessibility between ML- and SC-stabilized nanodispersions can be partially attributed to the difference in the structure of the emulsifier within the digestion period. Unlike ML, SC undergoes hydrolysis in the simulated stomach fluid containing pepsin (protein digestive enzyme), and this reduces their ability to prevent particles from growth and precipitation after small intestinal digestion (Agboola and Dalgleish 1996, Qiu *et al.*, 2015, Mun *et al.*, 2015). In addition, the simple mixed micelles formed by bile salts and phospholipids from the bile extract may not be capable of solubilizing the ergocalciferol molecule released from SC-stabilized nanodispersions effectively. The higher bioaccessibility for nanodispersions stabilized by ML was presumably associated with the stronger ability of this emulsifier to inhibit ergocalciferol particles from aggregation at the end of the digestion period. Another possible explanation for the relatively higher ergocalciferol bioaccessibility for ML-stabilized nanodispersions is that

ML formed relatively smaller particle size than SC in the initial delivery system. Pervious study has shown that the initial particle size of dispersion significantly influenced the micelle formation efficiency for β-carotene, with larger particle size resulting in lower bioaccessibility (Wang *et al.*, 2012a). Fig. 5.6 also indicated that the presence of milk significantly improved the ergocalciferol bioaccessibility (increased to ≈ 82%) for both ML- and SC-stabilized nanodispersions. Milk, known as a natural oil-in-water emulsion, can be digested within the small intestine phase containing pancreatin, and then release the free fatty acids and monoacyglycerols from the lipid phase. The fatty acids, monoacyglycerols, bile salts and phospholipids in the small intestinal fluid can form complex mixed micelles, which significantly increase their capacity for solubilizing the hydrophobic ergocalciferol (Mun *et al.*, 2015, Zou *et al.*, 2016).

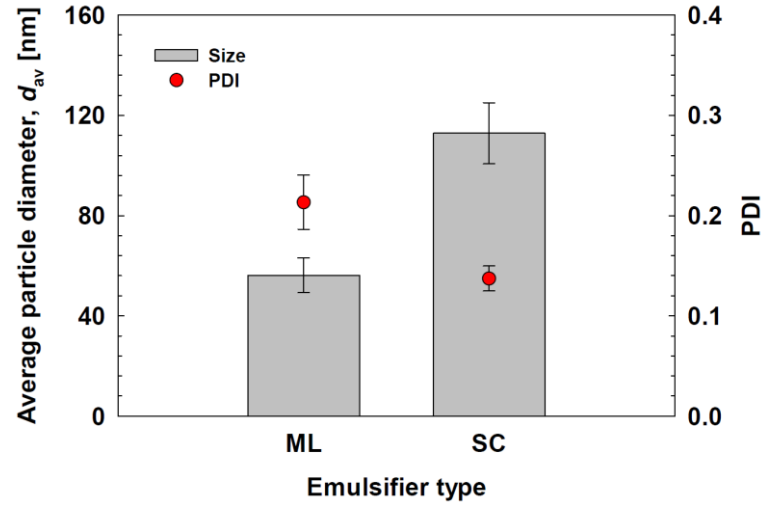
We also evaluated the chemical stability of ergocalciferol in the end of the *in vitro* gastrointestinal digestion process (Fig. 5.7). In general, the degradation of ergocalciferol was relatively low for all samples, with more than 83% of the initial ergocalciferol remaining at the end of the simulated intestinal digestion. It is well known that vitamin D is quite sensitive to decomposition when exposed to acidic conditions or elevated temperatures (Khalid *et al.*, 2015, Grady and Thakker 1980). Therefore, the slight loss of ergocalciferol found in our study is most likely due to the acid-degradation in gastric phase and thermal-degradation (37 ºC) during the whole digestion period. According to Fig. 5.7, the extent of ergocalciferol decomposition was slightly slowed down under simulated gastrointestinal conditions when the nanodispersions was incorporated with milk. We believe the lesser loss of ergocalciferol for the nanodispersions in the presence of milk was related to the iron-chelating and anti-oxidative properties of the high level of peptides released from the milk protein under simulated gastrointestinal conditions (Tan

et al., 2016c).

5.4 Conclusions

In the current study, nanodispersion-based delivery systems for ergocalciferol were successfully fabricated via solvent displacement method using ML and SC as emulsifiers. In comparison with SC, ML was a more effective emulsifier at forming nanodispersions containing smaller particles. SC-stabilized nanodispersions were unstable to particle growth when near the isoelectric point (pH 4 and 5) of protein, heating and in the presence of $CaCl_2$ addition. However, instability with slight increase in particle size were observed in ML-stabilized nanodispersions only when they were added with $CaCl_2$ solution. Both ML- and SC-formed nanodispersions exhibited good physical and chemical stabilities during storage at 4 ^{o}C for a period of 30 days. The study of *in vitro* gastrointestinal digestion demonstrated that in the absence of milk, the emulsifier type could affect the ergocalciferol bioaccessibility of the nanodispersions. Our results also indicated that the presence of milk in nanodispersions could significantly improve the bioaccessibility of ergocalciferol after digestion.

(a)



(b)

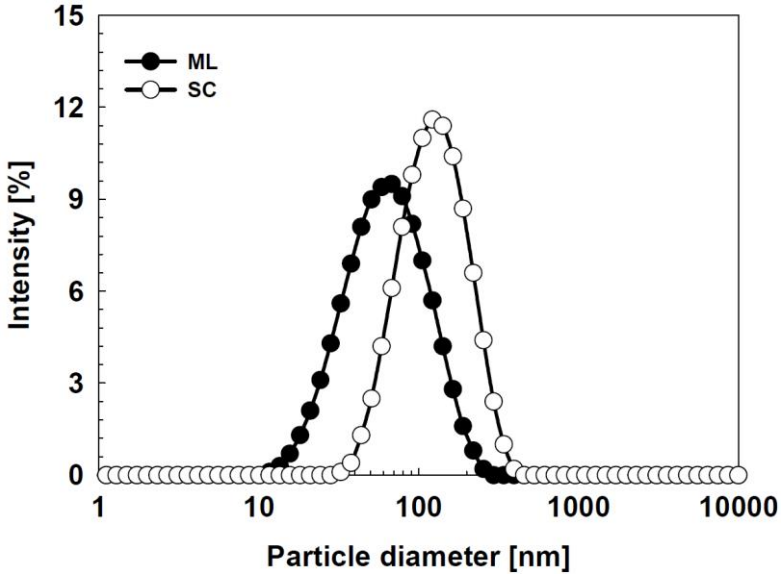
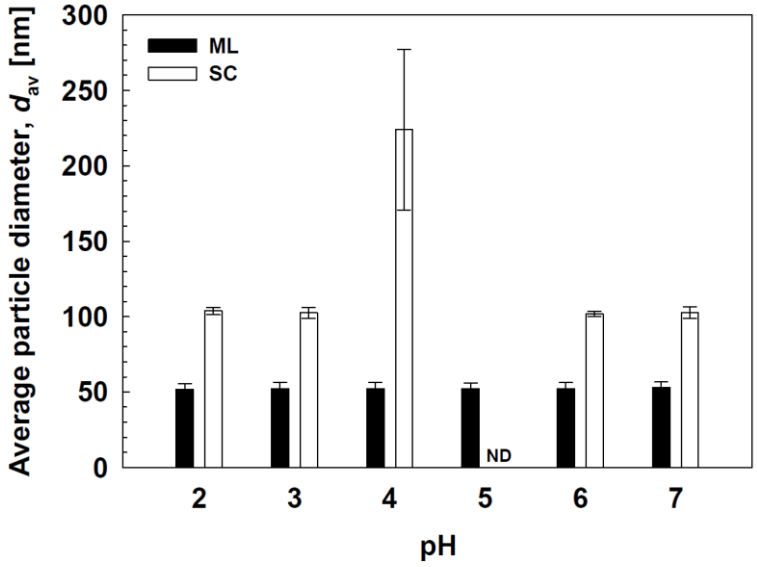


Fig. 5. 1: Effect of emulsifier type on the formation of ergocalciferol nanodispersions via solvent displacement method. (a) Z-average mean diameter (d_{av}) and polydispersity index (PDI) of nanodispersions stabilized by ML and SC.

(a)



(b)

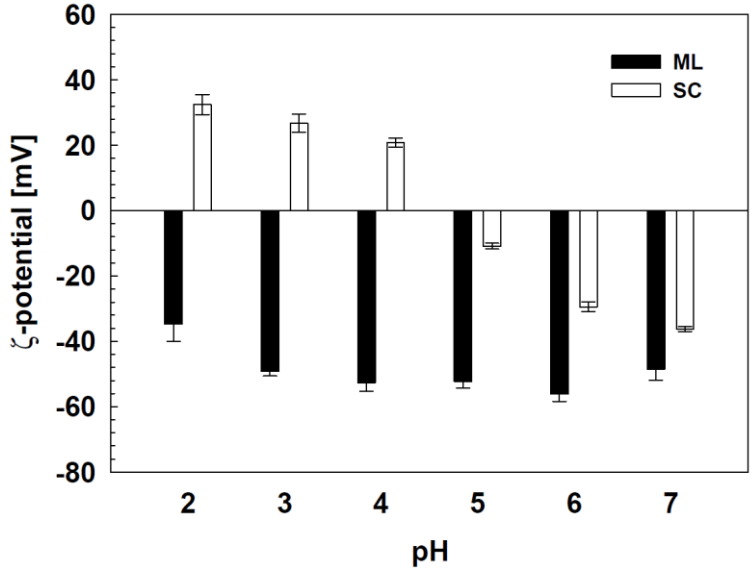
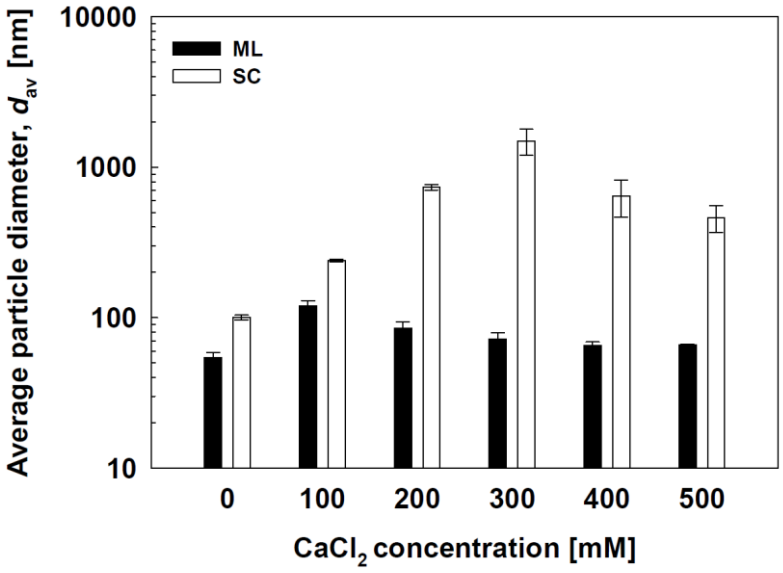


Fig. 5. 2: Effect of pH on the stability of ergocalciferol nanodispersions. (a) d_{av} and (b) ζ-potential of the nanodispersions. ND means not determination, because of the sediment of particles.

(a)



(b)

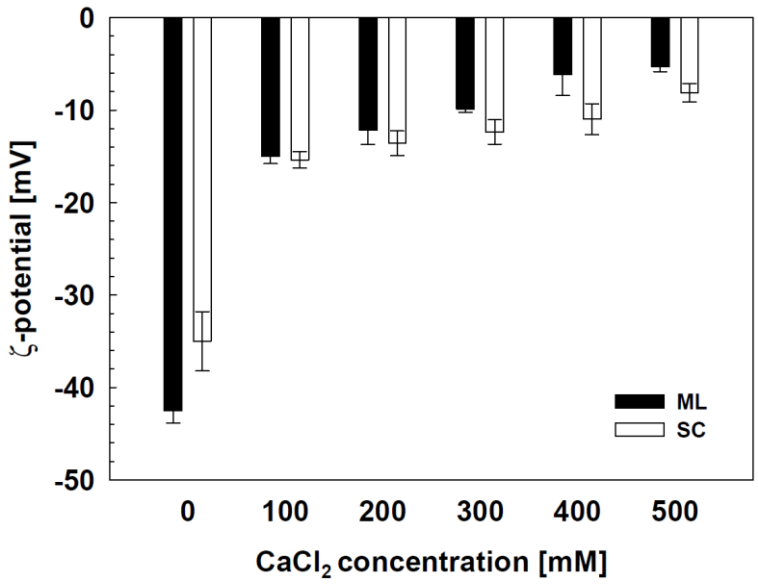


Fig. 5. 3: Effect of $CaCl_2$ addition on the stability of ergocalciferol nanodispersions. (a) d_{av} and (b) ζ-potential of the nanodispersions. Samples were diluted using water (1:19 (v/v)) prior to ζ-potential measurements.

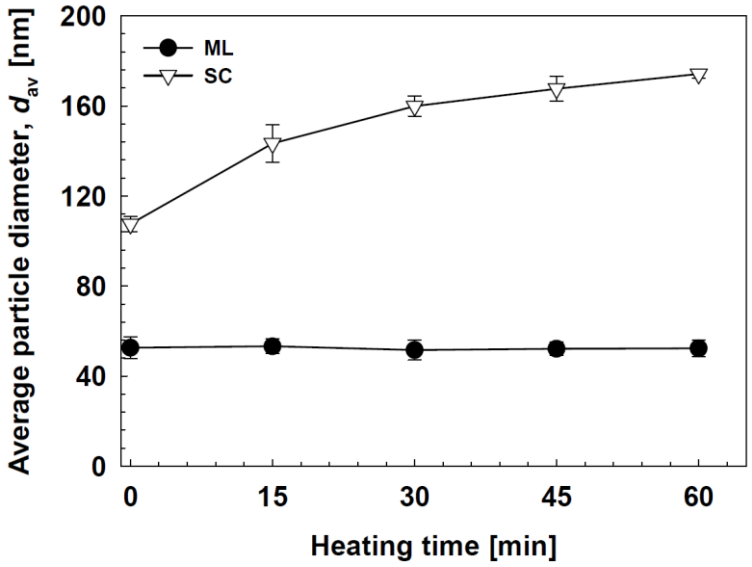
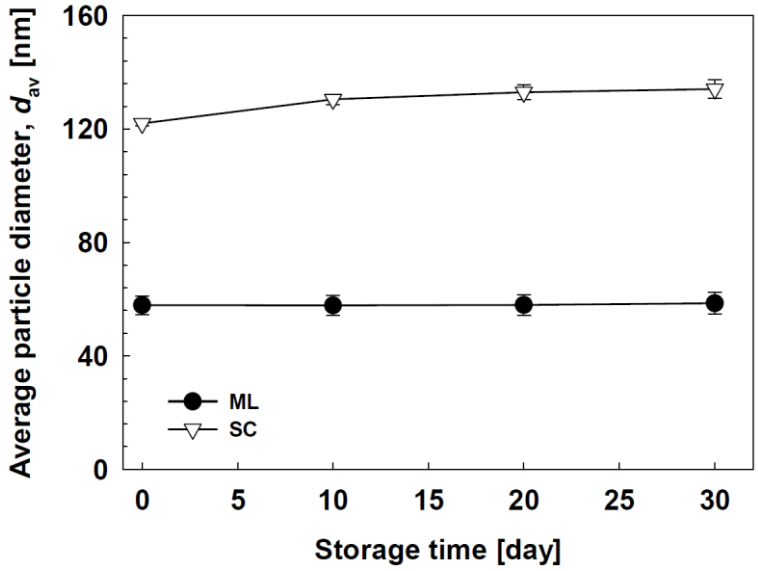


Fig. 5. 4: Effect of heating time on d_{av} and stability of ergocalciferol nanodispersions. The heating temperature was set at 120 °C.

(a)



(b)

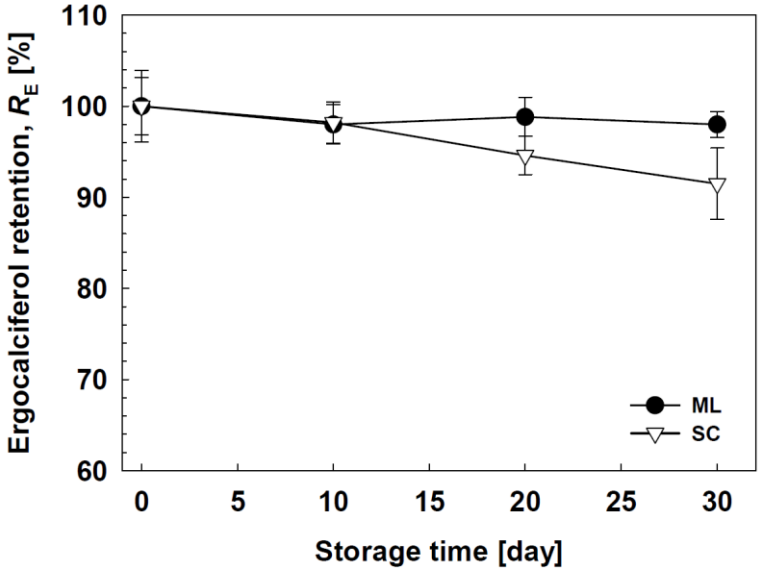


Fig. 5. 5: Long-term storage stability of ergocalciferol nanodispersions. (a) Physical stability and (b) Chemical stability of nanodispersions during 30 days of storage at 4 °C.

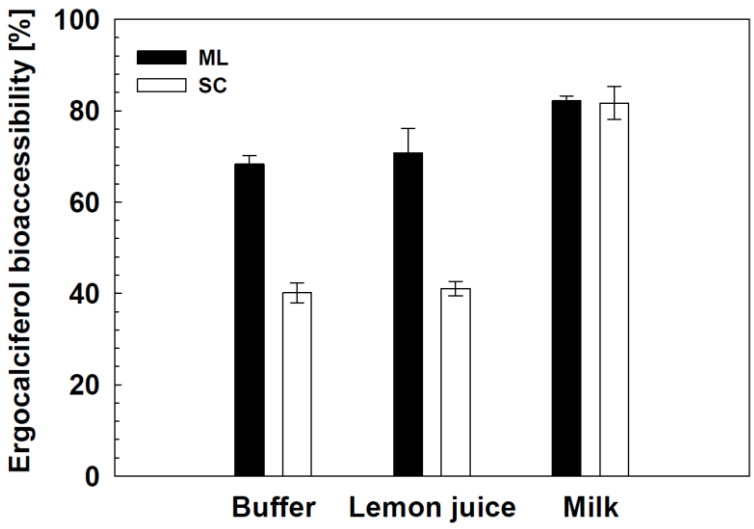


Fig. 5. 6: Bioaccessibility of ergocalciferol nanodispersions after *in vitro* gastrointestinal digestion. The *in vitro* gastrointestinal digestion behavior was studied in buffer solution, milk and lemon juice.

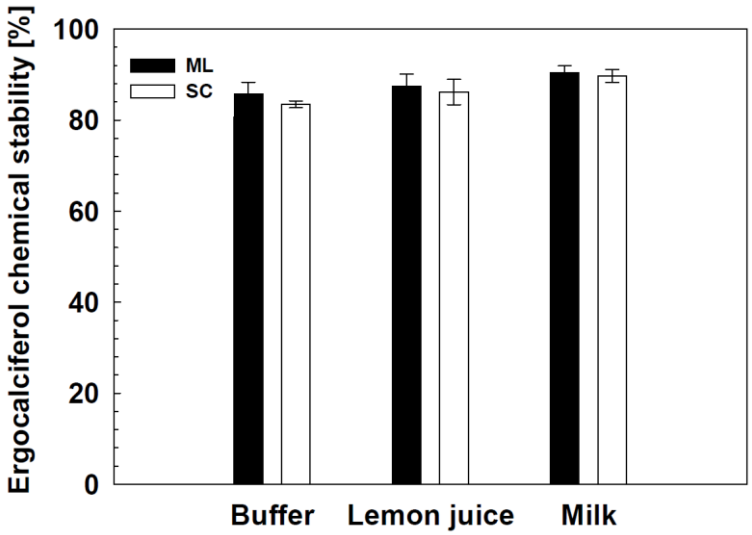


Fig. 5. 7: Chemical stability of ergocalciferol nanodispersions stabilized by ML and SC after *in vitro* gastrointestinal digestion. The *in vitro* gastrointestinal digestion behavior was studied in buffer solution, milk and lemon juice.

Chapter 6

General Conclusions and Future Prospective

Vitamin D deficiency is prevalent in many populations, which results in adverse health effects. Thus, it is important to develop foods and beverages with this water-insoluble bioactive compound. This study therefore concentrated on the developing effective dispersion systems for encapsulating and delivering ergocalciferol (vitamin D_2). To better understand this dissertation, each chapter of this thesis was summarized in the following part.

6.1 Summary of each chapter

6.1.1 Chapter 1

In this part, fundamentals of vitamin D, nanoemulsion/nanodispersion-based delivery systems were reviewed. The objectives and outlines of this thesis were also described

6.1.2 Chapter 2

In the part, ergocalciferol-loaded nanoemulsions were prepared by using high-pressure homogenization method. The effects of emulsifier type and concentration, oil type and concentration, and homogenization pressure on the droplet characteristics of nanoemulsions produced by high-pressure homogenizer were investigated. The results showed that the average size of emulsified droplets decreased with increasing operating pressure and emulsifier concentration. Nano-sized droplets ($d_{4,3}$ < 150 nm) could be successfully formed using soybean oil, perilla oil and MCT. The nanoemulsionss stabilized by modified lecithin (ML), sodium caseinate (SC) or decaglycerol monooleate (MO7S) showed similar droplet size and size distribution.

6.1.3 Chapter 3

In this part, the effect of emulsifier type on the stability of ergocalciferol-loaded nanoemulsions was investigated. The stability of resulting nanoemulsions was evaluated when they exposed to different environmental stresses and during 30 days of storage at 25 and 55 ^{o}C. Results showed that the emulsions prepared by MO7S or ML were stable against a wide range of pH (2-8), while SC-stabilized emulsions showed instability with extensive droplet aggregation at pH 4 and 5. Only ML-stabilized emulsions showed droplet growth due to coalescence when treated at high NaCl concentration (300-500

mM). In the absence of glucose, SC-stabilized O/W emulsions showed better freeze-thaw stability, in comparison to those formed with ML or MO7S emulsifiers. The emulsion produced by ML was found to be stable to droplet aggregation at high temperatures (80-120 ^{o}C) for 1 h. All the O/W emulsions stored at 25 ^{o}C showed good physical and chemical stabilities. However, the chemical stability of ergocalciferol in emulsion system decreased in order of ML > MO7S >> SC during storage at 55 ^{o}C for a period of 30 days. Overall, we are positively confident that the findings obtained from this study will be important and useful for future designs of nano-delivery systems to encapsulate lipophilic functional compounds, such as vitamin D.

6.1.4 Chapter 4

In this part, the effect of emulsifier type on the in vitro bioaccessibility of ergocalciferol-loaded nanoemulsions was examined (mouth, stomach and small intestinal phases). Results indicated that the droplet size, size distribution, ζ-potential and microstructure of nanoemulsions during digestion depended on the emulsifier type. The fate of lipid in the small intestinal phase also relied on the emulsifier type, with the free fatty acids release rate decreasing in the following order: MO7S > ML-MO7S > ML > SC. The ergocalciferol bioaccessibility in nanoemulsions prepared using MO7S, ML, and ML-MO7S was 62%, 64%, 65%, respectively, which was higher than that stabilized by SC, 12%. No significant loss of ergocalciferol was observed in all nanoemulsions after full digestion; they were chemically stable against digestion conditions, regardless of the emulsifier type.

6.1.5 Chapter 5

In this part, the formulation, stability and bioaccessibility of ergocalciferol nanodispersions stabilized by ML and SC as natural emulsifiers were investigated. The mean particle size of nanodispersions stabilized by ML, 56 nm, was much smaller than those stabilized by SC, 112 nm. The ML-stabilized nanodispersions were stable over a wide range of pH, NaCl concentrations and heating, but became unstable with slight increase in particle size when exposed to $CaCl_2$ solution. In comparison, SC-stabilized nanodispersions were relatively unstable, becoming aggregation under the conditions of pH 4-5, $CaCl_2$ addition and heating. Long-term stability for ergocalciferol were observed

in both ML- and SC-stabilized nanodispersions. The ergocalciferol bioaccessibility was strongly dependent on the emulsifier type, with ML providing much higher bioaccessibility than SC.

6.2 General conclusions

- Ergocalciferol-loaded O/W nanoemulsions with relatively small droplet size ($d_{4,3}$ < 150 nm) could be successfully prepared by high-pressure homogenization method.
- Both ML and SC could be used as a natural emulsifier to produce ergocalciferol nanodispersions via solvent displacement method.
- Ergocalciferol nanodispersions formed via solvent displacement method could be stabilized by low concentration of emulsifier.
- Environmental stresses play an important role on the stability of the resulting ergocalciferol nanodispersions.
- Efficient nanodispersion-based delivery systems with high stability and ergocalciferol bioaccessibility were prepared, but were highly dependent on emulsifier type.
- Each emulsifier has its own advantages and disadvantages, emulsifier selection should be talked case by case.
- ML-stabilized nanodispersions are promising delivery systems for ergocalciferol, and they have potential application for fortifying many food products.

6.3 Future prospective

In the present work, we chose to work with *in vitro* digestion model because it was a simple and straightforward method to evaluate the bioaccessibility of encapsulated bioactive compounds in dispersions. We hope the data obtained from our study could provide some important mechanistic insights into the performance of ergocalciferol nanoemulsion/nanodispersions prepared with different emulsifiers when exposed to

simulated conditions of the gastrointestinal tract (GIT). However, the behavior of those emulsified systems under real gastrointestinal conditions are still unknown. Thus, future study are required to have a more detailed investigation based on in vivo studies, using animal or human models, to determine the correlation between in vitro and in vivo digestion. In this study, we focused on the study of nanoemulsions and nanodispersion-based delivery system for encapsulating ergocalciferol. However, there are many other type of nano-delivery system that can be used for food supplication. Thus, it would be meaningful to have a systematic comparison of different delivery systems, in order to select the most suitable and efficient way for ergocalciferol encapsulation.

We are positively confident that the findings obtained from this study will be important and useful for future designs of nano-delivery systems to encapsulate lipophilic functional compounds, such as vitamin D. Food fortification with vitamin D contributes to human health by providing this micronutrient to prevent a series of diseases. A variety of products (e.g., soups and bervages) could be fortified with vitamin D-enriched nanoemulsions/nanodispersions, which may solve vitamin D deficiency prevalent in many people, e.g., old women who are suffering from osteoporosis.

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